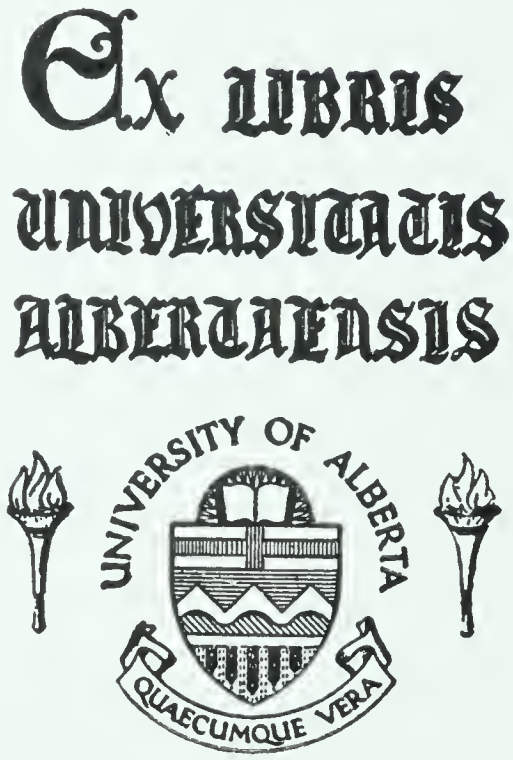

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NUTRITIONAL STUDIES AND CHARACTERIZATION OF THE PIGMENT OF MICROCOCCUS VIOLAGABRIELLAE

A DISSERTATION

Submitted to the Faculty of Graduate Studies

in Partial Fulfilment of the Requirements

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MASTER OF SCIENCE

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by

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FACULTY OF GRADUATE STUDIES

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled "Nutritional Studies and Characterization of the Pigment of Micrococcus violagabriellae" submitted by Jack Loran Nichols, B.Sc., in partial fulfilment of tne requirements for the degree of Master of Science.

ABSTRACT

The environmental and nutritional requirements for growth and pigmentation in Micrococcus violagabriellae were studied.

The effect on growth and pigmentation of oxygen, inorganic and organic nitrogen sources, carbohydrates and vitamins were determined. A dependence of pigmentation on iron was demonstrated.

The physiological significance of the pigment is discussed. It is suggested that the organism be placed in a taxonomic position with the staphylococci.

The pigment of M. violagabriellae was isolated and compared with other major groups of bacterial pigments. Its peptide, carbohydrate and iron components were studied.

On the basis of IR and UV spectra, solubility characteristics and inhibitor studies the pigment was described as being melanin-like, but possessing characteristics common to pulcherrimin.

A comparison was made of the pigment of M. violagabriellae with 'melanin' pigments isolated from other microorganisms.

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INTRODUCTION

Pigmentation (Chromogenesis)

A large number of widely distributed microorganisms produce non-photosynthetic pigments. Essentially all the colors of the visible spectrum are represented in microbial pigments.

In some pigment-producing species, the pigment is retained within the cell, whereas for others the pigment is excreted and colors the medium.

In general, pigments are produced only in the presence of oxygen and pigmentation occurs best on solid rather than liquid media. For strong pigment production special media are usually required and in some cases, mineral salts.

A temperature lower than that required for optimal growth is usually necessary for maximum pigmentation. As the temperature increases, pigment production decreases.

Although most bacterial pigments are carotenoids, other groups such as anthocyanins, melanins, porphyrins, tripyrrylmethenes and phenazines are frequently encountered in microbial systems.

The physiological significance of most pigments remains obscure (Williams, 1956). Some microbial pigments are antibiotics such as pyocyanin from Pseudomonas

aeruginosa, iodinin from Chromobacterium iodinum and chlortetracycline from Streptomyces aureofaciens.

Because pigment production generally takes place in the presence of oxygen, some believe that they act as respiratory carriers. Certain Pseudomonas pigments stimulate oxygen uptake of nonpigmented cultures, and it has been suggested that these pigments might have a respiratory function (Williams, 1956).

Origin of Micrococcus violagabriellae

In 1955, Castellani reported the isolation of a micrococcus from an erythmatic dermatitis which he described as being very close in taxonomic position to Staphylococcus aureus and Staphylococcus albus.

The most evident differentiating characteristic was a violet pigmentation of the colonies on glucose agar and potato. Pigmentation of the cultures on the agar was influenced by the variety of peptone employed in the preparation of the medium. With peptones of American origin, the pigmentation was very weak.

Castellani (1955) proposed the name Micrococcus violagabriellae nov. sp. for the organism isolated.

CHAPTER I

NUTRITIONAL CHARACTERISTICS AND ENVIRONMENTAL CONDITIONS AFFECTING GROWTH AND PIGMENTATION OF MICROCOCCUS VIOLAGABRIELLAE

CHAPTER I

A. Introduction

Together with the object of preparing a synthetic medium for culturing Micrococcus violagabriellae, studies on the nutritional characteristics and environmental conditions affecting growth and pigmentation of the organism were attempted in order to determine the relationships existing between this organism and other Micrococcus and Staphylococcus species.

In addition, it was thought that the chemical identification of the pigment would be facilitated by a study of the requirements for pigment synthesis.

B. Materials and Methods

1. Culture

The organism employed in these studies was Micrococcus violagabriellae, obtained from the American Meat Institute Foundation, Chicago.

2. Media Employed

a) Basal Medium

Most of the nutritional studies employed basal medium containing the following constituents in the final concentrations indicated per 100 ml final volume of media.

Casamino acids (Difco)	1.0 g
KH_2PO_4	0.5 g
$Na_3C_6H_5O_7 2H_2O$ (sodium citrate)	0.5 g
NaCl	0.5 g
$C_6H_{12}O_6$	0.5 g
Salts C	2.0 ml
Ferric concentrate	2.0 ml
Yeast extract (Difco)	0.02 per cent

The final volume was obtained by the addition of distilled water. Prior to sterilization for fifteen minutes at 121 C, the pH was adjusted to 7.2 with KOH.

Ferric concentrate was prepared by the addition of 0.5 g of $FeCl_3$ to 100 ml of distilled water.

Salts C were prepared as follows:

$MgSO_4.7H_2O$	4.0 g
NaCl	0.2 g
$MnCl.4H_2O$	0.72 g
Distilled water	100 ml

(Baltimore Biological Laboratory)

Trypticase soy broth (B.B.L.) was prepared according to instructions and sterilized by autoclaving.

Solid media was obtained by the addition of 1.5 per cent Difco agar to the medium prior to sterilization.

b) Media for Determining Carbohydrate Requirements

To ascertain the carbohydrate requirements for the organism, basal medium was employed in which the glucose was omitted. L-aspartic acid and L-cystine were substituted for the casamino acids in the growth experiments, but not in the pigmentation experiments.

All carbohydrates studied were added to the medium to a final concentration of 0.028 M.

c) Media for Determining Amino Acid Requirements

Casamino acids were omitted from the basal medium, and individual amino acids or groups of amino acids were substituted.

Stock solutions of L-amino acids (Nutritional Biochemical Corporation) were prepared and added to a final concentration of 0.4 mg/ml.

d) Media for Determining Vitamin Requirements

In the vitamin experiments, the same quantity of vitamin-free casein hydrolyzate was substituted for casamino acids in the basal medium.

Vitamins were prepared in stock solutions and diluted to yield the final concentration indicated below.

Vitamin	Concentration in Media
Biotin	0.01 γ/ml
Folic acid	0.01 γ/ml
Nicotinic acid	0.5 γ/ml
Pantothenic acid	0.1 γ/ml
Riboflavin	0.1 γ/ml
Thiamine	0.1 γ/ml
Pyridoxine	0.01 γ/ml

3. Preparation of Inoculum

Inoculum was prepared by washing the growth off a 24 hour culture grown on Trypticase soy agar. The inoculum was washed three times with sterile distilled water. Between the second and third washing, the cells were incubated at 37 C for 1 hour to reduce endogenous metabolism.

4. Techniques Employed

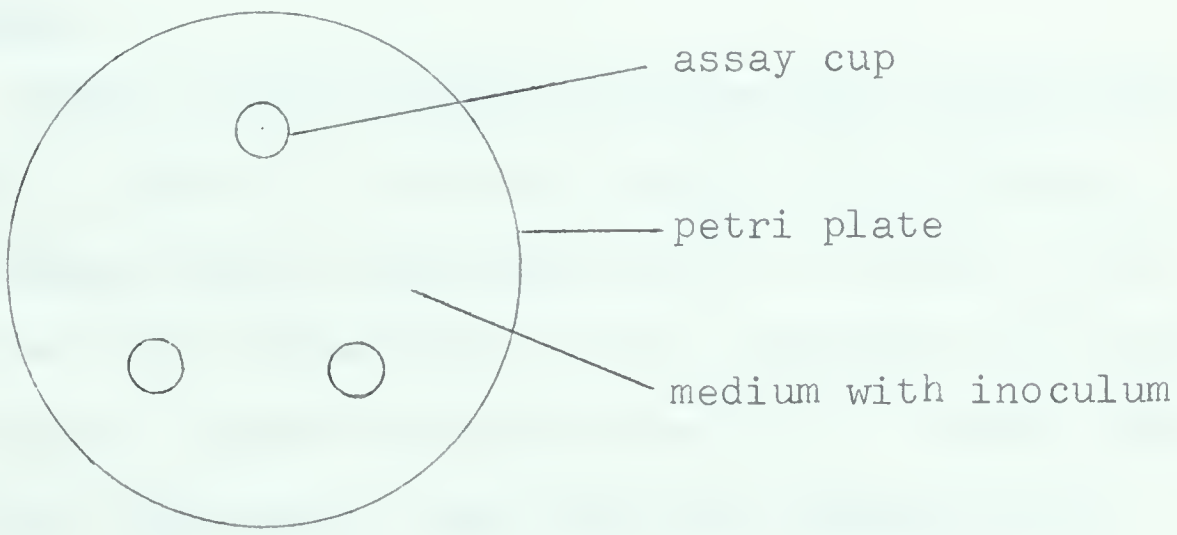
Growth experiments were carried out in standardized cuvettes, each of which contained a final volume of 10 ml. In most cases the basal medium was made up in double strength, the test compound was then added, and the final volume was made to 10 ml using distilled water.

Prior to autoclaving, the pH of each tube was adjusted to 7.2 and aluminum caps were used to close the cuvettes.

Acid cleaned glassware was used throughout the nutritional studies.

The ability of the organism to synthesize pigment was determined using two methods. The first of these comprised the streaking of inoculum on agar plates containing the various test compounds. The second was an auxanographic technique whereby the test compound was placed in an assay cup embedded in the agar. The inoculum was incorporated directly into the medium. Using this technique, several compounds could be tested on the same plate, in separate assay cups, or together in one cup.

The diagram below illustrates this method.



Unless otherwise designated, all cultures were incubated aerobically at 30 C.

5. Estimation of Growth Response

Growth response was determined turbidimetrically by taking optical density (O.D.) readings on a Bausch & Lomb Model 340 Spectronic 20 colorimeter.

Viable counts were made by adding a constant dilution of inoculum onto the plates and counting the colonies after incubation.

6. Expression of Results

Most of the results in the growth experiments were expressed as percentage increase in growth, and were calculated by the following formula:

$$\frac{\text{O.D. of test solution - O.D. of control}}{\text{O.D. of control}} \times 100 = \text{Percent Increase Growth}$$

In experiments designed to test the growth response to vitamins, the control consisted of a mixture of all 7 vitamins in the media (with concentrations indicated previously). The growth obtained in this control tube was designated as 100 per cent. The effect on growth of eliminating vitamins singly or in groups from the control was expressed as per cent relative growth.

C. Results

1. Effect of Temperature on Growth and Pigmentation

Table I (page 10) shows the result of incubation temperatures on growth and pigmentation. Optimal growth was obtained at a temperature of 37 C, whereas the optimal temperature for pigment synthesis was 30 C. Growth occurred over a wider temperature range than did pigmentation.

TABLE I

Effect of Incubation Temperature on Growth and Pigmentation of M. violagabriellae

Time	Incubation Temperature (C)	Growth Response	Pigment Production*
24 Hours	6	-	-
	20	-	-
	25	++	-
	30	+++	++
	37	++++	+
48 Hours	6	-	-
	20	(+-)	-
	25	+++	-
	30	++++	++++
	37	++++	+
72 Hours	6	-	-
	20	+	-
	25	+++	-
	30	++++	++++
	37	++++	++

* Estimated visually.
++++ represents maximum response
- represents no response
(+-) represents barely discernible response

Duplicate slants of Trypticase soy agar containing 100 γ/ml $FeSO_4$ were employed.

2. Effect of Oxygen on Growth and Pigmentation

The growth of M. violagabriellae is facultative with respect to oxygen, but pigmentation requires free atmospheric oxygen. Pigmentation was evident when cells were grown on solid media, and only in liquid media if the cultures were continually aerated.

3. Effect of Various Salts on Pigmentation

To determine their effect on pigmentation, various salts were separately added (to a concentration of 10 mg/100 ml) to Trypticase soy agar.

Of the cations and anions tried, only iron stimulated pigmentation and then only in concentrations above those required for optimal growth. $FeCl_3$, $FeCl_2$, $FeSO_4(NH_4)_2SO_4.6H_2O$, $FeSO_4.7H_2O$, $Fe_2(SO_4)_3NH_2O$, and ferric ammonium citrate would satisfy this iron-requirement for pigment synthesis.

4. Effect of Iron Concentration on Pigmentation

To ascertain a level of iron necessary for maximum pigmentation, cells were inoculated on Trypticase soy agar plates containing 0, 50, 100 and 1000 γ/ml $FeCl_3$. Pigment response was estimated visually.

The results summarized in Table II (page 12) show that maximum pigmentation on agar plates is evident on the addition of 100 γ/ml of iron to the medium. The addition of higher levels of iron to the medium had no visible effect on pigment synthesis and the growth response remained constant with the addition of high levels of iron necessary for pigmentation.

TABLE II

Effect of Iron Concentration on Pigmentation of M. violagabriellae

Concentration of Iron (γ/ml)	Pigmentation*	Growth Response (Viable Cells/ml)
0	(+-)	10.3×10^8
50	+	11×10^8
100	++++	9.6×10^8
1000	++++	10.9×10^8

* Estimated visually.

++++ represents maximum response

(+-) represents barely discernible response

Cultures were incubated at 30 C for 48 hours.

Although there was no diffusion of pigment into the medium, it was noted that when pigmenting cells were washed off agar plates or slants, the agar was stained the color of the pigment (Figure I) (page 14). This indicates that the pigment is released from the cells, but fails to diffuse because of its insolubility.

5. Effect of Nitrogen Source on Growth and Pigmentation

a) Inorganic Nitrogen

Table III (page 15) illustrates to what extent 2 nitrogen salts, NH_4Cl and $NaNO_3$ were utilized for growth. A concentration of 1 per cent NH_4Cl in the medium resulted in a growth response equal to that obtained with some amino acids (Table IV) (page 16). The organism is, however, not able to use nitrate as $NaNO_3$ as a sole nitrogen source, but is capable of reducing nitrates to nitrites (Campbell, 1963).

NH_4Cl and $NaNO_3$ were separately added to basal medium in varying concentrations. Agar plates were then streaked with the organism. Both salts failed to support pigmentation even after 96 hours of incubation.

b) Organic Nitrogen (Amino Acids)

Table IV (page 16) shows the effect of individual amino acids on the growth of M. violagabriellae. The ability to support growth was much greater with some amino acids than with others.

In order to ascertain the least number of amino acids which could be added to a synthetic medium to obtain good growth, amino acids were eliminated in groups from media containing all the amino acids.

FIGURE I

Staining of Medium by Pigmented Cultures of

M. violagabriellae

TABLE III

Effect of Inorganic Nitrogen Salts on the Growth of *M. violagabriellae*

Salt Added to Basal Medium	Growth Response (O.D. at 600 mu)*	Per Cent Increase Growth
None	.173	0
0.1% NH_4Cl	.268	54.9
0.4% NH_4Cl	.283	63.5
1.0% NH_4Cl	.318	83.8
1.0% $NaNO_3$	.183	5.7

* Readings were made of duplicate tubes and the mean value recorded.

Incubated at 37 C for 24 hours.

TABLE IV

Effect of Individual Amino Acids on the Growth of *M. violagabriellae*

Amino Acid Added to Basal Medium	Growth Response (O.D. at 600 mu)*	Per Cent Increase Growth
None (Control)	0.270	0
L-aspartic acid	0.473	75.2
L-cystine	0.401	48.5
L-histidine	0.398	47.4
L-alanine	0.383	45.6
L-cysteine	0.389	44.0
L-threonine	0.380	43.7
L-serine	0.380	40.7
L-arginine	0.358	32.6
L-glutamic acid	0.347	28.5
L-leucine	0.347	28.5
L-tyrosine	0.336	24.4
L-glycine	0.335	24.0
L-lysine	0.323	19.7
L-tryptophan	0.318	17.8
L-valine	0.303	12.2
L-phenylalanine	0.303	12.2
L-methionine	0.282	4.4
L-isoleucine	0.260	-3.7
L-proline	0.249	-7.7

* Readings were made of duplicate tubes and the mean value recorded.

Cultures were incubated at 30 C for 48 hours.

Table V (page 18) demonstrates that no appreciable decrease in growth resulted from the elimination of amino acids until only 6 amino acids were present. This combination of amino acids which supported best growth were L-aspartic acid, L-cystine, L-histidine, L-alanine, L-cysteine, and L-threonine (group b).

No direct dependence of pigmentation on any one amino acid could be demonstrated. As can be seen from Table V (page 18), pigmentation could be correlated with growth response. That is, the more amino acids that were added to the medium, the greater was the growth response and pigmentation.

6. Effect of Carbohydrates on Growth and Pigmentation

Each of the carbohydrates was separately tested for its ability to support growth. Table VI (page 19) shows that the organism was able to utilize several carbohydrates, but the growth response was greatest on fructose. Neither the trisaccharide raffinose nor the polysaccharides were utilized by the organism.

The ability of the carbohydrates to support pigmentation was tested by a combination of two methods. The basal medium containing only one carbohydrate was streaked with inoculum from an actively growing culture, and the auxanographic technique was used (Figure II) (page 20). In these experiments, unlike those dealing with the growth response, casamino acids were present in the medium.

TABLE V

Effect of the Addition of Groups of Amino Acids on Growth and Pigmentation of M. violagabriellae

Amino Acids Added to Basal Medium*	Growth Response (O.D. at 600 mu)**	Per Cent Increase Growth	Pigmentation***
None	.155	0	-
All amino acids in Table IV	.64	313	++++
Group a amino acids	.62	300	++++
Group b amino acids	.61	294	+++
Group c amino acids	.45	190	(+-)

* Group a amino acids: L-aspartic acid, L-cystine, L-histidine, L-alanine, L-cysteine, L-threonine, L-serine, L-arginine, L-glutamic acid and L-leucine.

Group b amino acids: L-aspartic acid, L-cystine, L-histidine, L-alanine, L-cysteine and L-threonine.

Group c amino acids: L-aspartic acid, L-cystine, and L-histidine.

** Cultures were incubated at 30 C for 48 hours.

Readings were made of duplicate tubes and the mean value recorded.

*** Estimated visually.

++++ represents maximum response
(+-) represents barely discernible pigmentation
\- represents no pigmentation

TABLE VI

Effect of Carbohydrates on Growth and Pigmentation of *M. violagabriellae*

Carbohydrate Added to Basal Medium	Growth Response (O.D. at 600 mu)*	Per Cent Increase Growth	Pigmentation**
None	.02	0	-
D-glucose	.280	1300	+
L-fructose	.340	1600	++++
D-galactose	.165	725	-
D-arabinose	.095	375	-
D-ribose	.120	500	++
D-xylose	.105	425	-
Sucrose	.175	775	+++
Lactose	.094	370	-
Maltose	.288	1340	+
Raffinose	.057	185	-
Inulin	.06	200	-
Dextrin	.015	-25	-
Starch	.008	-60	-
Salicin	.018	-10	-

* Cultures were incubated for 48 hours at 30 C. Optical Density readings were made of duplicate tubes and the mean value recorded.

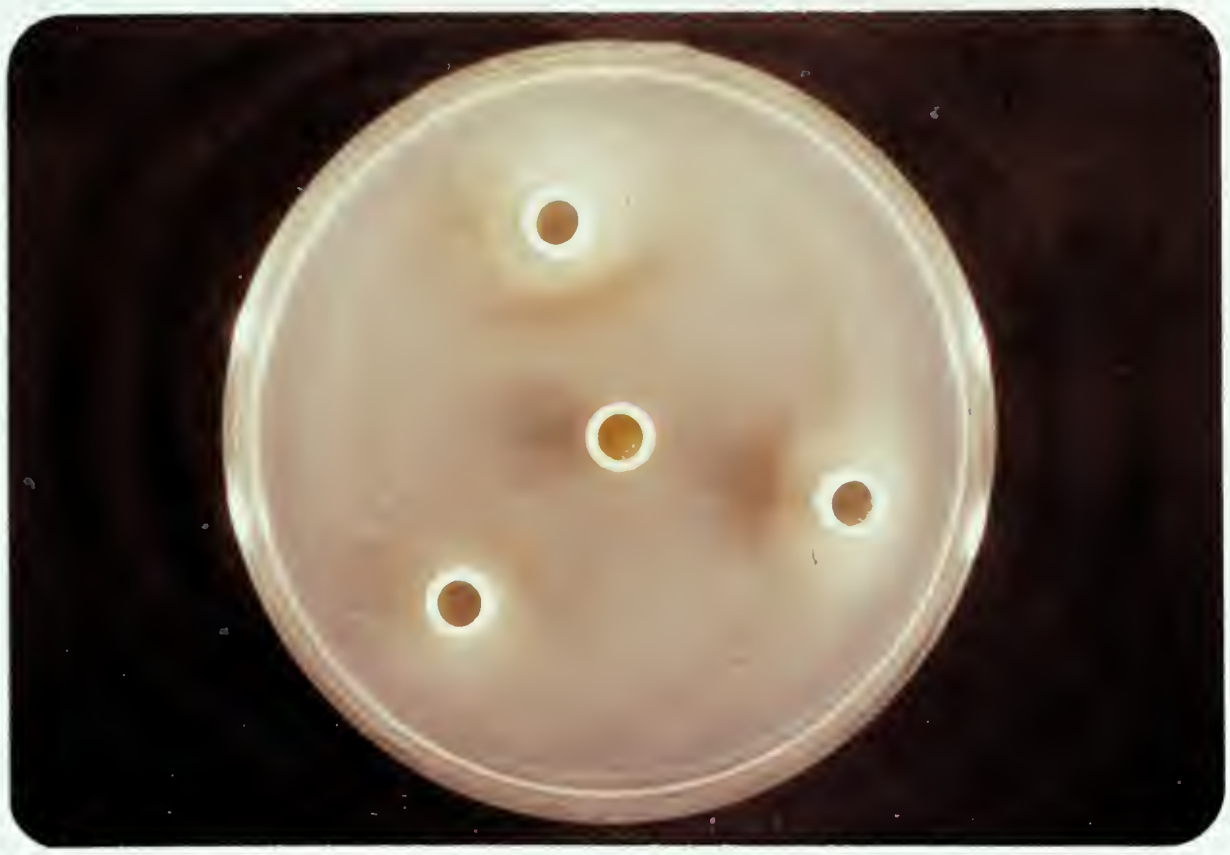
** Estimated visually.

++++ represents maximum response

\- represents no response

FIGURE II

Response of M. violagabriellae to the Presence of Different Carbohydrates*



* Center assay cup - iron ($FeCl_3$)
Top assay cup - fructose
Left assay cup - sucrose
Right assay cup - glucose

The ability of fructose to support maximal growth of M. violagabriellae is coincident with its ability to support maximal pigmentation (Table VI) (page 19). Whereas glucose and maltose were more important than ribose and sucrose as carbohydrate sources for growth response, the latter two compounds supported pigmentation to a greater extent. As was expected from the growth studies, the trisaccharide and the polysaccharides were not utilized in pigment synthesis.

7. Effect of Vitamins on Growth and Pigmentation

In order to determine the relative importance of each vitamin, the subtractive technique was employed in which a vitamin was omitted from the basal medium containing a group of vitamins.

Table VII (page 22) shows an absolute dependence of growth on nicotinic acid and a high dependence on thiamine and biotin. None of the vitamins tested was inhibitory to growth.

Table VIII (page 23) illustrates the fact that nicotinic acid alone cannot support growth and only poor growth resulted from its combination with biotin and thiamine separately.

The group of 3 vitamins which, in combination, supported best growth were: nicotinic acid, biotin and thiamine (designated as 'basal group').

It was found that increasing the concentrations of vitamins in the 'basal group' either separately or simultaneously had no effect on the growth response.

TABLE VII

Effect of Vitamin Deprivation on Growth Response in *M. violagabriellae*

Vitamin Omitted from Basal Medium	Growth Response (O.D. at 600 mu)*	Per Cent Relative Growth
None	.60	100
Biotin	.30	50
Folic acid	.34	57
Nicotinic acid	.053	8
Pantothenic acid	.428	71
Thiamine	.145	24
Pyridoxine	.423	70
Riboflavin	.49	82

* Cultures were incubated at 30 C for 72 hours.

Optical density readings were made of duplicate tubes and the mean value recorded.

TABLE VIII

Effect of Added Vitamins on

Growth and Pigment Production by M. violagabriellae

Vitamins Added to Basal Medium	Per Cent Relative Growth	Pigmentation*
All vitamins	100	++++
Nicotinic acid	11	-
Nicotinic acid Biotin	12	-
Nicotinic acid Thiamine	23	+
Nicotinic acid Biotin Thiamine	47	+++

* Estimated visually.

++++ represents maximum response

- represents no response

Incubated at 30 C for 48 hours.

By adding vitamins separately to basal medium agar, it was found that no single vitamin by itself could support pigmentation. Nicotinic acid, biotin and thiamine were found to be necessary to support pigmentation equal in intensity to that produced when all the vitamins were present (Table VIII) (page 23).

Although the pigment produced by the addition of the 'basal group' to the medium was an intense red color, the appearance of growth on the plate was different. Instead of a confluent growth, there appeared numerous, isolated colonies of various sizes. These colonies appeared drier and the surface, instead of being smooth, was rough. Since this colonial morphology persisted when subcultured on Trypticase soy agar with iron, there is a possibility that a mutant was being selected.

Microscopic examination of the cells grown in the presence of these vitamins failed to reveal any morphological differences.

D. Discussion

Micrococcus violagabriellae, in view of the fact that it is facultatively anaerobic, can best be placed in the genus Staphylococcus, according to the proposed scheme of Evans, Bradford, and Niven (1955).

It differs from the staphylococci in the presence of an iron-dependent red pigment, its ability to utilize inorganic nitrogen as NH_4Cl (to some extent at least) and the fact that it is insusceptible to Staphylococcus phage

29, 52, 52A, 79, 80, 3A, 3B, 3C, 55, 71, 6, 7, 47, 53, 54, 42E, 73, 75, 77, 81, 82, 187 and 42D. Furthermore, it differs from most pathogenic staphylococci in that it is coagulase negative (Campbell, 1963).

M. violagabriellae grows best at a temperature of 37 C. This is not surprising in view of the fact that Castellani (1955) isolated the organism from a tropical superficial dermatitis. The optimal temperature for growth does not correspond to the optimum for pigmentation. This is characteristic of most pigment synthesizing systems where the optimal pigment-synthesizing temperature is lower than the temperature at which growth occurs best (Williams et al., 1958; Haynes et al., 1956).

The temperature range which will allow pigmentation is, in the strain studied, smaller than that which will allow the growth of the organism.

Cassingena, Ortali, Villa and Milazzo (1960) found M. violagabriellae grew between 20 and 44 C and that the production of pigment occurred between 22 and 40 C. On this basis, it is conceivable that the organism used by Cassingena et al. is a different strain from the one studied here.

The finding that the organism is facultatively anaerobic is in agreement with the work of Castellani (1955) and Cassingena et al. (1960).

The synthesis of the colored pigment is dependent on a ferric or ferrous salt in the medium. Cassingena et al. (1960) independently showed this iron requirement for pigmentation.

Growth does not require the high concentrations of iron needed for pigmentation. Thus the only nutritional requirement necessary in high concentrations for pigmentation, but apparently not for growth, is iron. The absence of this metal is probably the reason Castellani could not observe the pigment when growth was carried out utilizing peptones of American origin.

When grown on basal medium agar or Trypticase soy agar, staining of the medium took place, but the pigment did not diffuse into the medium. The pigment must therefore be released from the cells, but it does not diffuse into the medium because of its insolubility.

Inorganic nitrogen as NH_4Cl, and most of the amino acids were found to be stimulatory for growth. No absolute requirement for an amino acid either for growth or pigmentation could be demonstrated. Instead, a group of 6 amino acids were found to be necessary for optimal growth and pigmentation.

Stocchi (1956; quoted in Cassingena et al., 1960) working with strains of M. violagabriellae found that the organism did not utilize ammonium in the form of $NH_4H_2PO_4$ but was capable of using some organic compounds as a nitrogen source. Here again there is evidence for strain differences.

Several carbohydrates satisfied the carbon requirements of the organism both for growth and pigment synthesis. The optimal response was produced on fructose. These results are in agreement with those of Cassingena et al. (1960).

The only absolute growth-requiring substance which could be demonstrated in the organism was nicotinic acid. As the only vitamin present in the medium, however, nicotinic acid could not support growth. To obtain relatively good growth and pigmentation, nicotinic acid, biotin and thiamine had to be placed in the basal medium.

All of the vitamins tested were stimulatory to the organism, since the elimination of any one from the group of vitamins resulted in a drop in the growth response.

Because of the many differences existing between growth and pigment requirements (both environmental and nutritional) in this organism, and because pigmentation is not necessary for the growth of physiologically sound organisms, it seems probable that the processes dealing with the growth of the organism are distinct from those involved in pigment synthesis. However, this does not exclude the possibility of common metabolic precursors or intermediates.

DeMoss and Happel (1959) state that "in some respects pigment formation may be considered to be a luxury operation by the bacterial cell". Since growth in the absence of pigmentation appears normal, this may be the case in

M. violagabriellae. This suggestion for the non-functional role of pigments has also been put forward in Williams (1956).

Payne and Campbell (1962) found that pigmented cells of M. violagabriellae are more resistant to UV irradiation than non-pigmented cells. In view of their findings, pigment synthesis in this organism may be of great physiological significance.

E. Conclusions

1. The strain of M. violagabriellae studied herein differs from those of Cassingena et al. and Stocchi, in that it was able to utilize an inorganic nitrogen salt, its temperature range for pigmentation was narrower, and maximum pigmentation was obtained after 24 hours.

2. The dependence of pigmentation, but not growth, on oxygen and high levels of iron was determined.

3. Both inorganic (as NH_4Cl) and organic nitrogen sources were stimulatory to growth, but to obtain an optimal growth and pigmentation response, at least six amino acids had to be added to the basal medium. No absolute requirement for any single amino acid could be demonstrated. Inorganic nitrogen salts could not support pigmentation.

4. Several carbohydrates could satisfy the requirements for growth and pigmentation; optimal response was obtained with fructose.

5. All the vitamins studied were stimulatory for growth and pigment synthesis, and nicotinic acid was essential.

CHAPTER II

STUDIES ON THE PIGMENT OF *MICROCOCCUS VIOLAGABRIELLAE*

CHAPTER II

A. Introduction

Studies on the isolated pigment were carried out to determine the nature of the pigment and its components, and thereby to attempt to place it in one of the major groups of bacterial pigments.

B. Materials and Methods

1. Medium Employed for Pigment Production

The basal medium used for the production of pigment contained the following constituents in final concentrations indicated per 100 ml final volume of medium.

Casamino acids (Difco)	1 g
Cysteine	10 mg
Tryptophan	10 mg
Aspargine	10 mg
KH_2PO_4	0.5 g
$Na_3C_6H_5O_7 2H_2O$ (sodium citrate)	0.5 g
NaCl	0.5 g
$C_6H_{12}O_6$	0.5 g
Yeast Extract (Difco)	0.03 per cent
Salts C	2 ml
Ferric concentrate	2 ml

Salts C and Ferric concentrate were prepared as outlined in Chapter I.

The medium was made up to volume with distilled water and the pH adjusted to 7.2 using KOH. If solid media was required, 1.5 per cent Difco agar was added. Sterilization was for 15 minutes at 121 C, or by filtration using a Millipore filter size 0.45 u.

2. Isolation of Pigment

Agar plates inoculated heavily with the organism were incubated for 72 hours at 30 C and refrigerated for 12 to 24 hours.

The growth was washed off the plates with distilled water and centrifuged. The pigment-cell mass was then treated with N KOH whereupon the red pigment became soluble and changed to a bright yellow color. The yellow solution was centrifuged for 30 to 40 minutes at 20,000 x G, the supernatant removed and centrifuged once again.

Following this, the clear yellow solution was neutralized with HCl whereupon the red pigment precipitated. Finally, the pigment was washed 3 times with distilled water, and dried in a vacuum desiccator.

When further purification was required, the alkali-acid conversion was repeated several times.

3. Tests for the Major Groups of Pigments

a) Carotenoids (Mrak, Phaff, and Mackinney, 1949)

Pigmented cells were suspended in a test tube containing 10 ml of 1:1 HCl-water. The mixture was heated to a boil and promptly cooled.

After cooling, 10-15 ml of acetone and 3-5 ml of petroleum ether were added and the mixture shaken. Water (15-20 ml) was then added to effect a clear-cut separation of the two phases.

If carotenoids were present, the upper (epiphasic) petroleum ether layer was colored.

b) Porphyrins (Granick, 1958)

Alkali-acid purified pigment was extracted 3 times, each time with 20 ml of ethyl acetate - acetic acid (3:1 v/v) followed by centrifugation.

The combined extract was neutralized with sodium acetate and washed with water. The porphyrin was extracted from the ethyl acetate layer with a total of 5 ml of 2.5 N HCl.

Absorbancy was measured in the regions between 548 and 556 mu or 400 and 409 mu.

c) Melanins

i) Ammoniacal Silver Nitrate

$AgNO_3$	10 g
Distilled water	100 ml
Concentrated NH_4OH	Added until solution cleared

Pigment was added to the above solution and observed for the precipitation of silver.

ii) Schmorl Method (Pearse, 1960)

Pigment	several mg
1 per cent $FeCl_3$	3 parts
1 per cent potassium ferricyanide	1 part

Substances such as melanins which reduce ferricyanide to ferrocyanide appear dark blue.

4. Solubility Characteristics of the Pigment

To determine the solubility of the pigment in its native state, portions of a cell-pigment paste were placed either on a spot plate or in a test tube and the solvent to be tested was added.

The solubility of the alkali-acid prepared pigment was tested by placing a few crystals on a spot plate and adding the solvent.

5. Chromatography: Solvent Systems and Location Reagents

Unless otherwise indicated, Whatman No. 1 chromatography paper was used.

a) Solvent Systems

i) Water Saturated n-butanol (Smith, 1960)

n-butanol and distilled water were mixed in a separatory funnel. After standing for 2 hours, the lower water layer was placed on the bottom of the chromatography tank; the top butanol layer was used as the developing solvent.

ii) n-butanol - ethanol - ammonia (Smith, 1960)

n-butanol	7 ml
ethanol	1 ml
0.5 N NH_4OH	2 ml

iii) n-butanol - glacial acetic acid - water (Smith, 1960)

n-butanol	4 ml
glacial acetic acid	1 ml
distilled water	5 ml

The 3 components were mixed in a separatory funnel. The bottom layer was placed in the bottom of the chromatography tank and the top layer was used as the developing solvent.

iv) <u>iso-propanol - n-butanol - water</u> (Smith, 1960)

iso-propanol	140 ml
n-butanol	20 ml
distilled water	40 ml

v) <u>iso-propanol - water</u> (Smith, 1960)

iso-propanol	160 ml
water	40 ml

vi) <u>Toluene/Phosphate (2-dimensional)</u> (Smith, 1960)

First dimension:

toluene	5 ml
2-chloroethanol	3 ml
pyridine	1.5 ml
5 N NH_4OH	0.5 ml

Second dimension:

$NaH_2PO_4.2H_2O$	78 g
$NaHPO_4.2H_2O$	44.5 g
distilled water	1000 ml

vii) <u>Ethanol - benzyl alcohol</u> (Blackburn and Lowther, 1951)

Whatman No. 1 chromatography paper was first dipped in a 0.05 M phthalate buffer (pH=6) and dried.

0.1 M potassium hydrogen phthalate (20.418 g/l)	50 ml
0.1 N NaOH	45.45 ml
distilled water	to 100 ml

The solvent consisted of 10 per cent ethyl alcohol in benzyl alcohol. Before use, this solvent was saturated at room temperature with the same buffer that was applied to the paper.

viii) Small-scale filter paper technique

(Underwood and Rockland, 1954)

Chromatography paper employed was Schleicher & Schuell Blue Ribbon No. 589. The solvents were run ascending in a museum jar.

First dimension:

formic acid	10 ml
tertiary-butanol	695 ml
distilled water	295 ml

Second dimension:

phenol	775 g
distilled water	215 g

The phenol - water solution was stored at room temperature in a brown tightly-stoppered bottle. Prior to use, the phenol - water solution was mixed with concentrated NH_4OH in the proportion of 94 ml to 1 ml.

ix) Heptane - pyridine

heptane	70 ml
pyridine	30 ml

x) n-butanol - pyridine - water

n-butanol	65 ml
pyridine	65 ml
distilled water	65 ml

b) Location Reagents

i) Ninhydrin (Smith, 1960)

An 0.25 per cent solution of ninhydrin (triketo-hydrindene hydrate) in acetone was made. Chromatograms were sprayed and placed in an oven at 65 C for 2 to 3 minutes.

ii) Aniline - phthalic acid (Block, 1952)

aniline ..... 0.93 g
phthalic acid ..... 1.66 g
water saturated n-butanol ..... 100 ml

Chromatograms were sprayed and placed in an oven at 105 C for 5 minutes.

iii) Ammoniacal $AgNO_3$ (Block, 1952)

Chromatograms were sprayed with a solution consisting of a mixture of equal volumes of 0.1 N $AgNO_3$ and 5 N NH_4OH, and heated at 105 C for 5 to 10 minutes.

iv) Iodine - azide - starch (Smith, 1960)

Solution I:
iodine ..... 2.54 g
potassium iodide ..... 8 g
distilled water .....100 ml

Solution II:
sodium azide ..... 1.5 g
0.5% soluble starch ..... 100 ml

Solution II was prepared by mixing 0.5 g of soluble starch into a paste with 3 to 4 ml of cold water. This mixture was then poured into 90 ml of boiling water. The solution was allowed to cool, the sodium azide was added and the solution made up to 100 ml with distilled water.

Solution I and II were mixed in the ratio of 1:2 prior to use, and the chromatograms were dipped through the reagent.

6. Pigment Hydrolyzates

a) Twenty-two Hour HCl Hydrolysis

A 22-hour hydrolyzate of the pigment was prepared by adding 10-20 mg of the pigment to a thick-walled test tube. Approximately 3 ml of 6NHCl were placed in the tube and a small beaker placed over the mouth of the tube. The pigment was hydrolyzed in an autoclave at 121 C and 19 lbs pressure for 22 hours.

b) One-hour H_2SO_4 Hydrolysis

A one hour hydrolyzate was prepared in the same manner as the 22-hour hydrolyzate, except that 1.5 N H_2SO_4 was used and the reaction carried out for 1 hour at 100 C.

7. Melting Point Determinations

Melting points were determined on a Fisher melting point apparatus.

8. N-terminal Analysis

N-terminal analysis was carried out using two different methods.

a) Fluorodinitrobenzene (FDNB) Method (Kabat and Mayer, 1961)

A 10 per cent suspension of finely ground alkali-acid prepared pigment was made in 10 per cent aqueous $NaHCO_3$. This suspension was added to 2 volumes of a 10 per cent ethanolic solution of 1-fluoro-2,4-dinitrobenzene. The mixture was shaken for 2 hours at room temperature. The DNP-peptide (dinitrophenyl) was washed twice with each of water, ethanol

and ether. The remaining precipitate was dried and hydrolyzed for 24 and 4 hours in 6 N HCl.

The method in Smith (1960) was used to prepare known DNP-amino acids. One or 2 milligrams of the amino acid was dissolved in 1 ml of 2 per cent $NaHCO_3$ solution and mixed with 2 ml of ethanol containing 0.05 ml of 1-fluoro-2,4-dinitrobenzene. The mixture was shaken in the dark at room temperature for 2 hours. At the end of this time, the solution was diluted with 5 ml of water and extracted twice with peroxide-free ether. After acidification of the aqueous layer with 2 drops of 6 N HCl, the DNP-amino acid could be extracted by washing with 2 ml portions of ether. The combined ether extracts were washed with very dilute HCl and then evaporated to dryness.

To regenerate the amino acid from its unknown DNP-amino acid derivative, the latter was extracted from its hydrolyzate by means of ether. The amino acid was regenerated from this concentrated extract by heating with saturated barium hydroxide in a sealed tube at 100 C for 1 hour. Following this, the dissolved barium was precipitated as Ba_2CO_3 with CO_2 and the liquor evaporated to dryness.

b) Phenylisothiocyanate Method (Smith, 1960)

Approximately 20 mg of finely powdered pigment was dissolved in 5 ml of a pyridine - water mixture (1:1). The pH of the solution was adjusted to 9.0 and the solution was brought to 40 C in a water bath. 0.24 ml of phenyl-isothiocyanate was added and the tube shaken. The pH of the solution was maintained at 9.0.

After 1 hour, the excess reagent and pyridine was removed by 3 extractions with equal volumes of benzene.

The derivative was then dissolved in 5 ml of glacial acetic acid / HCl solution (18 ml of concentrate HCl made up to 100 ml with glacial acetic acid), and warmed to 37 C for 3 to 5 minutes, after which fission and cyclization was complete.

The solution was evaporated to dryness and the phenylthiohydantoin (PTH-derivative) was extracted with ethyl acetate.

Known PTH-derivatives were prepared as follows: To 1 gram of an amino acid, was added 25 ml of a 1:1 pyridine - water mixture. The procedure was then followed as for the preparation of the unknown except that NHCl was added after the benzene extraction in an amount equal to the volume of NaOH used to adjust and maintain the pH, plus 1 ml excess.

The derivative precipitated after partial evaporation of the solution.

Hydrolysis of the PTH derivative was accomplished by adding 1 ml of 5.7NHCl to the derivative and heating at 150 C in a sealed tube for 16-20 hours.

The acid was evaporated and a few drops of distilled water was added. The evaporation was carried out once more and the residue finally dissolved by the addition of 0.05 ml of distilled water.

9. Proteolytic Digestion

a) Trypsin

A tryptic digestion of the pigment (2 mg) was attempted in 0.03 M Tris buffer at pH 8.5. A concentration of 0.1 mg of enzyme / ml solution was employed. The incubation mixture was allowed to stand at 37 C for 48 hours with occasional shaking. The pigment was precipitated with acid, washed with distilled water 3 times and hydrolyzed with HCl for 22 hours (as indicated previously).

The hydrolyzate was examined chromatographically for the presence of amino acids.

b) Pepsin

Finely ground pigment (2 mg) was incubated in 0.03 M Tris buffer at pH 2.0, using a concentration of 1 mg / ml of pepsin. The mixture was incubated for 72 hours at a temperature of 37 C after which the pigment was washed, hydrolyzed with HCl and chromatographically examined as for the tryptic digest.

c) Peptidase

Pigment (2 mg) was dissolved in 0.03 M Tris buffer at a pH of 8.5. Peptidase to a final concentration of 1 mg / ml was added and the mixture incubated for 48 hours at 37 C. Acidification, washing, hydrolysis and chromatography were carried out as for the tryptic digestion.

d) Papain

Pigment (2 mg) was dissolved in 0.03 M Tris buffer at a pH of 8. Enzyme was added to a final concentration of 1 mg / ml. The solution was incubated for 24 hours at 50 C and

treated as indicated for the tryptic digestion.

10. Iron Determinations by the Dimethyl-p-phenylene-diamine Reaction (Ceriotti and Spandrio, 1961)

A weighed sample of pigment was dissolved in concentrated H_2SO_4, in the proportion of 400 mg of pigment to 1 ml of acid, and heated in a water bath at 60 C. The solution was diluted 1 to 4 with water. To 0.4 ml of this solution, 0.1 ml of HNO_3 and a boiling stone were added and the mixture was digested on a sand bath at 200 C. A few drops of hydrogen peroxide were added to clear the residue.

After the contents of the test tube was oxidized to a colorless solution, all the acids were evaporated on a small reducing flame, until only an ash remained.

To this ashed residue 2 ml of 2 N H_2SO_4 were added and the test tubes were heated for 15 minutes in a boiling water bath. After cooling, 1 ml of an acetate solution (8 g of sodium acetate dissolved in 10 ml of water) and 1 ml of water were introduced and the mixture was shaken.

One ml of dimethyl-p-phenylenediamine hydrochloride was added (0.1 per cent in 95 per cent ethanol) and the solution was thoroughly mixed.

The developed color was read at 515 mu against a blank treated in the same manner.

Ashed standards were prepared using analytical grade $NH_4Fe(SO_4)_2.12\ H_2O$.

11. Emission and Absorption Spectra

For emission spectrography, a Hilger Spectrograph (medium), employing a condensed AC spark, was used. Carbon electrodes with hollow ends were used to contain the sample.

Ultraviolet and visible absorption spectra were obtained on a Unicam SP.700 Spectrophotometer, unless otherwise indicated.

Infrared absorption spectra were obtained on a Model 21 Perkin-Elmer Infrared Spectrophotometer and a Perkin-Elmer Model 421 Spectrophotometer. A halolube 'mull' of the sample was made and the area from 5000 to 1300 cm^{-1} scanned. A nujol 'mull' of the sample was made and that area of the scale between 1300 and 650 cm^{-1} was scanned.

12. Spot Tests for Iron (Feigl, 1946).

The following spot tests were performed by dissolving the washed precipitate (from alkaline solution) in concentrate HCl and using this as the test solution.

a) Potassium Ferrocyanide

test solution ..... 1 drop
potassium ferrocyanide solution ..... 1 drop

The potassium ferrocyanide forms Prussian blue with ferric salts in acid solution. The compound can be reduced to white ferroferricyanide by reducing agents such as $Na_2S_2O_4$.

b) Potassium Thiocyanate

test solution ..... 1 drop
1 per cent potassium thiocyanate ..... 1 drop

Iron (Fe+++) ions combine with thiocyanate ions in acid to give a deep red color.

c) Thioglycolic Acid

test solution ..... 1 drop
thioglycolic acid ..... 1 drop
6 N ammonia ..... 1 drop

A purple-red color is produced in ammoniacal solution by the action of thioglycolic acid with iron (Fe++) and iron (Fe+++) salts.

13. Quantitation of Pigment

The following method was devised to quantitate pigmentation response to the addition of various compounds in the basal medium.

After washing of the cells 3 times with distilled water, varying proportions of the same optical densities of both pigmented cells and non-pigmented cells (p-cresol inhibited) were mixed. Five ml of each dilution was centrifuged and the pellet of pigment and cells was treated with 3 ml of N KOH. After centrifugation of these alkaline solutions, the optical densities of the supernatants were measured on the DU-Spectrophotometer at 413 mu.

The standard curve showed a linear relationship between increasing amounts of pigmented cells and the optical density at 413 mu.

Experiments to test the effect of various compounds on pigmentation were carried out by adding the compound to basal medium. After inoculation with an actively growing culture, flasks were placed on a shaker at 30 C for 48 hours. At the end of this time, the cultures were centrifuged and washed twice with distilled water.

To determine the effect on the growth response, optical density readings of a 1:40 dilution of washed cells were taken at 600 mu on a Bausch & Lomb Spectronic 20 Colorimeter.

Following this, each culture was made up to an optical density of 1.0, and 5 ml of this suspension was centrifuged and the optical density of the supernatant was determined on the DU Spectrophotometer at 413 mu.

14. Inhibitor Studies

Inhibitor studies employed filter-sterilized basal medium to which the test compound was added. The cultures were constantly aerated.

P-benzyloxyphenol was dissolved in methyl alcohol (0.5 ml / 20 ml basal medium) and the same amount of methanol was added to the control flask.

Phenylacetic acid was dissolved in 95 per cent ethanol (0.5 ml /20 ml basal medium) and the same amount of ethanol was added to the control.

15. Colorimetric Determination of 3,4-dihydroxyphenyl-alanine (Arnow, 1937)

One ml of an unknown solution (0.02 ml to 1 mg of dopa)

was placed in a test tube graduated to 5 ml. One ml of each of the following reagents was added in the following order:

- 0.5 N HCl
- Nitrite-molybdate reagent (10 g of sodium nitrite and 10 g of sodium molybdate in 100 ml distilled water)
- 1 N NaOH (to produce a red color)
- Enough distilled water to make a volume of 5 ml.

The resulting color was read at 510 mu.

16. Extraction of Bacterial 'Melanin' Pigments from Pseudomonas

The following procedure was used to extract and purify the pigments from the M 117 and MS strains of Pseudomonas aeruginosa.

Cultures were grown on Trypticase soy agar plates for 72 hours at 30 C. The agar from 5 to 10 plates was homogenized briefly in a Waring blender with distilled water. After this mixture had been in the water for 3 to 4 hours, it was filtered several times and centrifuged.

The resultant brown solution was evaporated to dryness, dissolved in a small quantity of water and filtered once more.

The filtrate was dialyzed overnight in running tap water and then poured on a column of DEAE-cellulose (diethylaminoethyl) (Selectecel Type 20) and washed with distilled water and 0.01 M NaCl. The pigment was eluted from the column with 0.05 M NaCl.

The eluate was dialyzed for 30 hours in running tap water and for 5 hours in distilled water.

The solution was evaporated to dryness in a vacuum desiccator.

C. Results

1. Tests for the Major Groups of Pigments

a) Carotenoids

Carotenoids are chemically characterized, in part, by their solubility in lipid solvents, resistance to attack by alkali, and the formation of a dark blue color with concentrated sulphuric acid (Fox, 1953).

The test for carotenoid pigments (Mrak, Phaff, and Mackinney, 1949) was negative when performed on the alkali-acid treated pigment.

None of these characteristics is shared with the pigment of M. violagabriellae, and it is therefore not a carotenoid.

b) Porphyrins

All porphyrins are soluble in glacial acetic acid, strong mineral acid, pyridine and dioxan (Falk, 1961). In addition, porphyrins can be extracted with ethyl acetate at an acid pH (Musilek, 1962).

These solubilities are not characteristic of the pigment isolated from M. violagabriellae (Table IX) (page 48).

The extraction procedure for porphyrins with less than 4 carboxyl groups (Granick, 1958) was negative. Absorption

TABLE IX

Solubility Characteristics of the Native Pigment and Alkali-acid Purified Pigment of M. violagabriellae

Solvent	Native Pigment*	Alkali-acid Prepared Pigment	Color in Solution
Distilled water	I	I	
Ethylene dichloride	I	I	
Benzene	I	I	
Isobutanol	I	I	
n-butanol	I	I	
Acetone	I	I	
Ethanol (95 per cent)	I	I	
Isopropanol	I	I	
Chloroform	I	I	
Methanol	I	I	
Pyridine	I	SS	Yellow
Dioxan	I	I	
2-chloroethanol	I	SS	Yellow
Dimethyl sulfoxide	I	I	
Dimethyl formamide	I	I	
Tetrahydrofuran	I	I	
Acetonitrite	I	I	
Phenol	I	I	
NHCl	I	I	
Concentrated HCl	S	S	Yellow-brown
NH_2SO_4	I	I	
Concentrated H_2SO_4	S	S	Brown
Glacial acetic acid	I	I	
Trichloroacetic acid	I	I	
N KOH	S	S	Yellow
N NaOH	S	S	Yellow
N NH_4OH	S	S	Yellow
Concentrated HNO_3	S	S	Yellow
Concentrated H_3PO_4	I	S	Pink
Perchloric acid	S	S	Brown
$NaHCO_3$ (10 per cent)	I	SS	Yellow

* I = insoluble
SS = slightly soluble
S = soluble

spectra revealed no Soret band around 400 mu, which is characteristic of porphyrins.

c) Anthocyanins

Anthocyanins are soluble in water and alcohol and are commonly extracted with methanolic - HCl. These solubility characteristics are not shared with M. violagabriellae pigment.

d) Phenazines

Both the native and alkali-acid prepared pigment were unlike pyocyanin (a phenazene pigment) in that they were insoluble in chloroform.

e) Tripyrrylmethenes

M. violagabriellae pigment is unlike prodigiosin in that it is insoluble in acetone.

f) Melanins

Melanins are characterized, in part, by their insolubility in common solvents other than strong aqueous alkali (with precipitation on the acidification of the solution), and reduction of ammoniacal silver nitrate. The characteristics are identical to those for the pigment of M. violagabriellae.

The addition of the alkali-acid prepared pigment to ammoniacal silver nitrate reduced the solution to yield black metallic silver with a resultant green-yellow solution.

The Schmorl method used to identify melanins was positive.

A further characteristic of many melanins, the bleaching of the pigment with an oxidizing agent such as permanganate or hydrogen peroxide, could not however be accomplished with this pigment.

2. Solubility Characteristics

Table IX (page 48) shows the solubilities of the pigment both in the native state and after alkali-acid purification. After treatment with alkali, the pigment is more soluble in pyridine, 2-chloroethanol (ethylene chlorohydrin), 10 per cent $NaHCO_3$ and concentrate H_3PO_4.

Solutions of the pigment in pyridine and ethylene chlorohydrin could not be precipitated by the addition of HCl or water. Evaporation of the ethylene chlorohydrin solution left a yellow precipitate.

The addition of water or alkali to a solution of pigment in concentrate HCl or 70 per cent perchloric acid resulted in the precipitation of the red pigment. However, the addition of water to a solution of pigment in concentrate H_3PO_4 resulted in a pink solution which remained stable.

After 24 hours, the addition of water to the HCl solution gave rise to a greatly reduced amount of precipitate.

After 72 hours, the amount of precipitate produced by the addition of water to the perchloric acid solution was reduced. Removal of the precipitate left a light brown solution, which could be extracted with n-butanol. Ether chloroform, benzene, carbon tetrachloride, ethylene

dichloride and toluene could not extract this material from acid solution.

Pigment dissolved in H_3PO_4 could not be extracted with n-butanol after the same length of time.

The lowest concentration of KOH which would dissolve the native pigment was 0.1 N, but to completely dissolve the pigment, the concentration of alkali had to be raised to 0.2 N.

Pigment in alkaline solution could not be extracted using chloroform, benzene, ethylene dichloride, isobutanol and n-butanol. Extraction of the alkaline solution could not be accomplished even if the pigment was neutralized to the greatest extent without causing precipitation.

When sonically treated native pigment was centrifuged, it was noted that the pigment was only partially precipitated and there remained a red-colored supernatant. On drying this solution, and performing solubility tests on the pigment, no differences could be detected from those in Table IX (page 48).

The pigment which was solubilized by sonic treatment was readily precipitated by acid.

3. Chromatography of Pigment

The alkali-acid prepared pigment, treated in the way indicated below, was chromatographed using water saturated n-butanol, n-butanol-ethanol-ammonia and n-butanol-glacial acetic acid-water solvent systems.

- red pigment
- pigment in N KOH
- pigment in N KOH, partially neutralized
- pigment dissolved in concentrated HNO_3, partially neutralized.

All the chromatographed pigments, with the exception of the red pigment and the HNO_3-treated pigment, when sprayed with ninhydrin, produced a deep-purple spot which was closely associated with a yellow component. These 2 components could not be separated and moved only very slightly from the origin.

None of the pigments exhibited more than 1 ninhydrin-positive spot nor more than 1 yellow component.

Each of the chromatograms was examined under ultraviolet light, but no UV absorbing or fluorescing components were seen.

4. Amino Acid Composition and N-Terminal Analysis

A 22-hour acid hydrolyzate was prepared of the alkali-acid treated pigment. Two methods were employed for the identification of the amino acids present.

The first method, a one-dimensional chromatographic system utilized n-butanol-acetic acid-water as the developing solvent. The second method was the 2-dimensional paper chromatographic procedure of Underwood and Rockland (1954) using solvent systems tert-butanol-formic acid-water and phenol-water.

Both methods, upon spraying with ninhydrin, revealed 6 amino acids.

Each of the amino acids was identified by running known amino acids beside the hydrolyzate, or directly with the hydrolyzate.

Amino acids present were indistinguishable from histidine, aspartic acid, glutamic acid, glycine, methionine and leucine by these techniques.

N-terminal amino acids were identified by the fluorodinitrobenzene and phenylisothiocyanate methods.

The amino acid derivative in the former method was identified by comparison with known derivatives. This was done in a 2-dimensional system (Underwood and Rockland, 1954) involving the 'toluene' solvent system. The ethanol-benzyl alcohol solvent system of Blackburn and Lowther (1951) was also used.

Both the 24 and 4-hour hydrolyzate revealed the presence of the same DNP-derivative: glycine.

Liberation of the amino acid from its derivative was followed by chromatographic comparison with a 22-hour hydrolyzate of the red pigment using n-butanol - acetic acid - water solvent. The amino acid was identified as glycine. This confirmed the earlier suggestion that there was but one N-terminal amino acid and that was glycine.

A PTH-derivative of the pigment obtained by the phenylisothiocyanate method was chromatographically compared with knowns using the heptane-pyridine solvent system and the iodine - azide starch location reagent.

Glycine was identified as the only PTH-derivative. Also, alkaline hydrolysis of the derivative released glycine.

5. Proteolytic Digestion, Acid and Alkaline Hydrolysis

Proteolytic digestion of the pigment was carried out using trypsin, pepsin, papain and peptidase. All these enzymes failed to separate the peptide component from the chromophore.

Acid and alkaline hydrolysis of the pigment carried out for varying lengths of time failed to separate the peptide component from the pigment without destroying the latter.

6. Carbohydrate Analysis

A 1-hour hydrolyzate of the pigment was prepared. Unlike the 22-hour hydrolyzate, it did not result in the decomposition of the pigment.

Chromatography of the hydrolyzate was carried out using isopropanol-n-butanol-water as the solvent. A pink spot, characteristic of a pentose, was located using the aniline-phthalic acid spray. Ammoniacal $AgNO_3$ location reagent confirmed the presence of this sugar.

By comparison with known pentoses, the sugar was tentatively identified as arabinose.

7. Quantitation of Iron

Iron determinations were carried out on the alkali-acid purified pigment to ascertain if iron was incorporated into the pigment.

The dimethyl p-phenylenediamine reaction of Ceriotti and $S_{pandrio}$ (1961) was used for the iron determinations. Using this method, values were obtained ranging from 0.20 per cent to 0.288 per cent iron, with an average value of 0.234 per cent iron.

However, a different sample of pigment purified in the same way showed only a trace of iron on analysis (see Part 8, below).

The treatment of the pigment with alkali must therefore quantitatively remove the iron.

Each of the following samples was examined for iron using emission spectrography.

I. Washed unpigmented cells (grown without iron in the medium)

II. Washed pigmented cells (grown with iron in the medium)

III. Precipitate from alkaline solution of pigment (see Part 12, below)

IV. Alkali-acid purified pigment (from same sample as used for analysis, Part 8, below).

Figure III (page 56) shows the emission spectrogram of samples I to IV. Known samples of iron appear above and below these 4 samples.

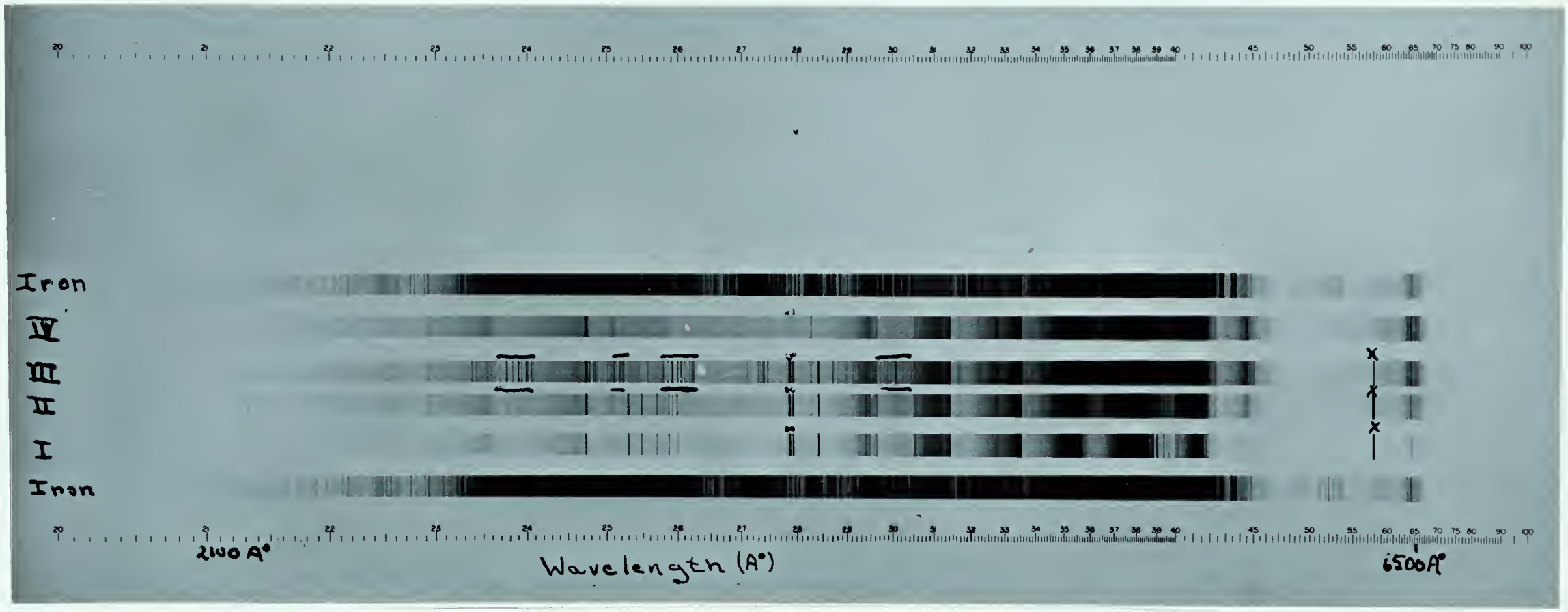
It can be seen that I and IV contain no iron, whereas samples II and III do contain iron. The concentration of iron in sample III is greater than in sample II.

These results illustrate the fact that iron is attached to the pigment, but purification involving alkali treatment removes it.

FIGURE III

Emission Spectrogram Illustrating

Iron Association of Pigment of M. violagabriellae



____ indicates iron absorbing areas

x indicates sodium absorbing areas

" indicates magnesium absorbing areas

8. Elemental Analysis of Pigment and Melting Point

Analysis of the alkali-acid purified pigment* revealed the following percentage composition:

54.24% C
8.32% H
8.48% N
0.85% S
A trace of iron
1.84% residue

Melting point of the pigment was above 300 C.

9. Dialysis of Pigment

Alkali-dissolved pigment, partially neutralized alkali-dissolved pigment and red pigment after sonic disruption (non-precipitable by centrifugation) were dialyzed overnight in running tap water.

The red pigment did not dialyze out but was retained within the dialysis casing.

The 2 different alkaline dialyzates were chromatographed using n-butanol-ethanol-ammonia as a solvent. No ninhydrin positive material could be detected on the chromatograms. On the addition of acid to these dialyzates, no precipitate formed.

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* Performed by Dr. Franz Pascher, Mikro-Analytisches Laboratorium, Bonn, Buschstrasze 54, Germany.

10. UV and IR Spectra

Alkali-acid purified pigment was used for all ultraviolet, visible and infrared spectra.

Figure IV (page 59) shows the spectrum of the pigment in N KOH. A broad peak between 227 and 246 mu is evident, with other peaks at 284 and 413 mu.

Figure V (page 60) is the IR spectrum of the red pigment and shows peaks at 3250, 2950, 1650 (broad), 1515, 1465, 1393, 1217, 1155, 1068, 998, 965, 950, 920 and 845 cm^{-1}.

The pigment in ethylene chlorohydrin exhibits a broad absorption peak at 221 mu with a shoulder from 240 to 270 mu and one further peak at 366 mu (Figure VI) (page 61).

The absorption spectrum of the pigment in H_3PO_4 exhibited absorption maxima at 274 and 370 mu with a broad peak at 475 mu (Figure VII) (page 62).

The spectrum of the perchloric acid treated pigment showed a single peak at 203 mu with no further peaks in the UV or visible range.

The spectrum of the pigment in concentrated HCl revealed a small peak at 223 mu with additional peaks at 273, 320 and 360 mu (Figure VIII) (page 63).

11. Observations on the Acid Precipitation of Pigment from Alkaline Solution. Comparison of the 'Red' and 'White' Pigment

Pigment dissolved in N KOH and left standing at room temperature gradually changed from a yellow to a colorless

FIGURE IV

UV and Visible Spectra of the Pigment of M. violagabriellae in Alkali

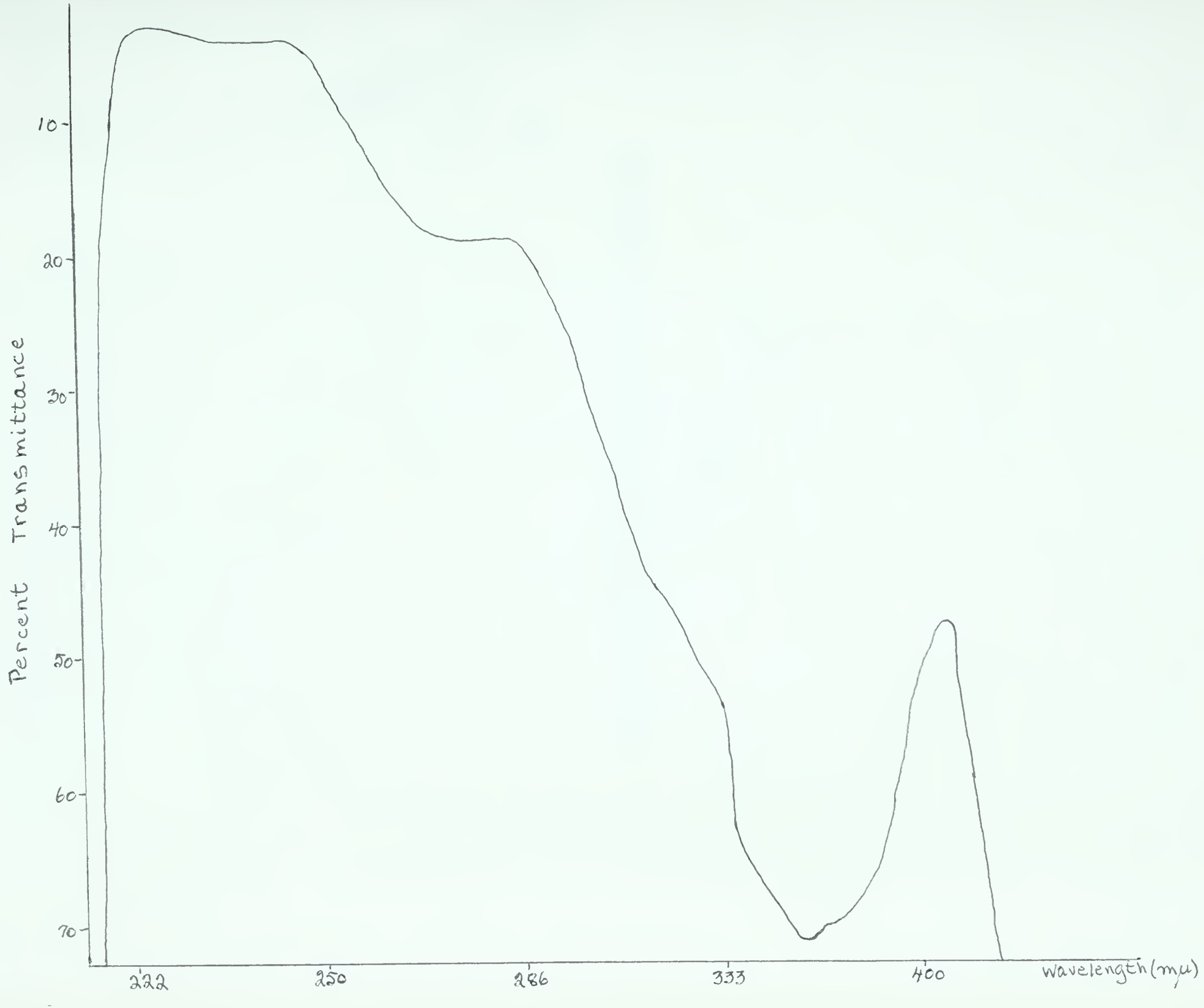
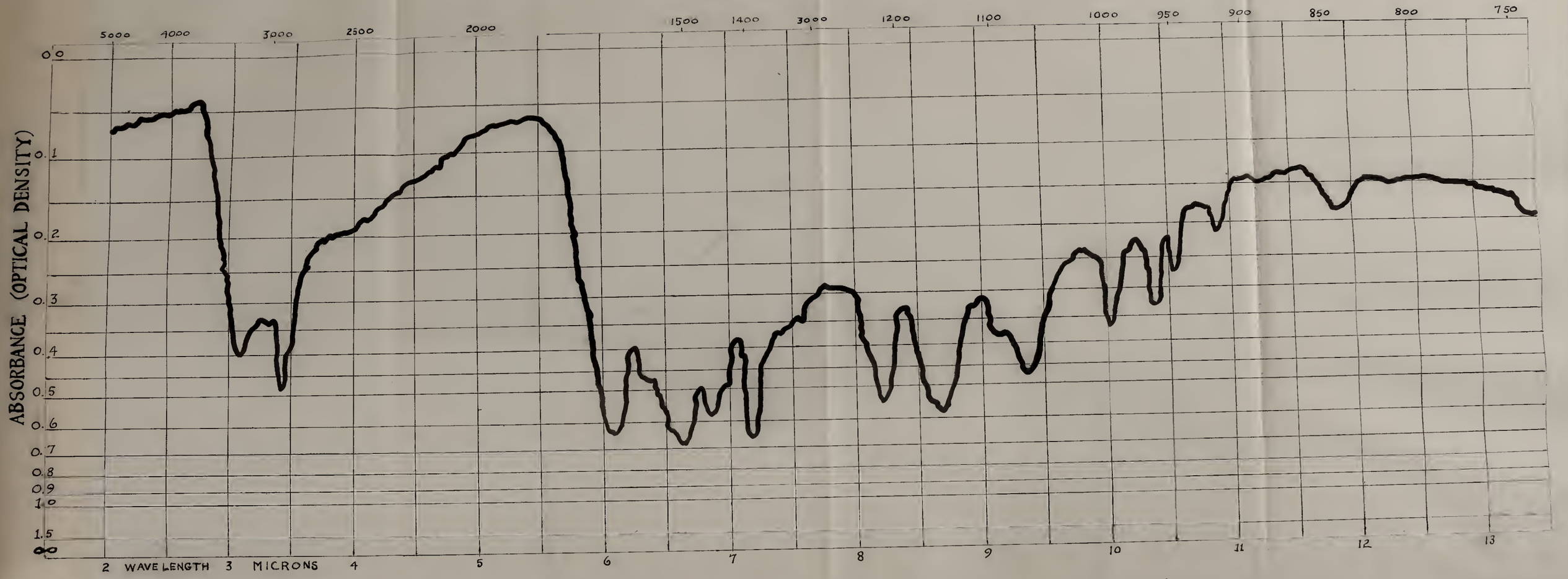

Percent Transmittance
10
20
30
40
50
60
70
222
250
286
333
400
wavelength (mμ)

FIGURE V

IR Spectrum of Pigment of M. violagabriellae


5000
4000
3000
2500
2000
1500
1400
3000
1200
1100
1000
950
900
850
800
750
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
1.5
∞
ABSORBANCE (OPTICAL DENSITY)
2 WAVE LENGTH 3 MICRONS 4
5
6
7
8
9
10
11
12
13

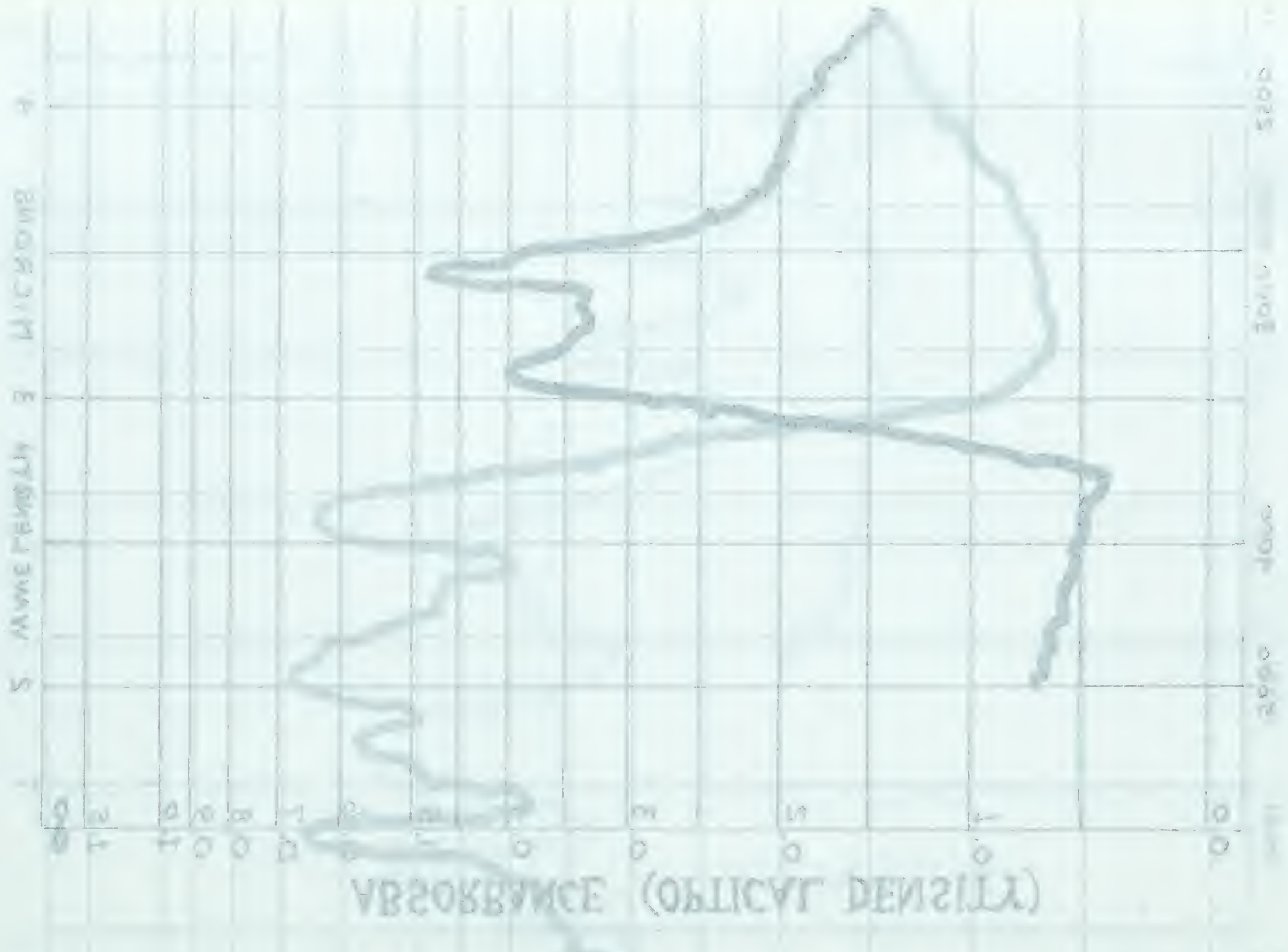

ABSORBANCE (OPTICAL DENSITY)

FIGURE VI

UV and Visible Spectra of the Pigment of
M. violagabriellae in Ethylene Chlorohydrin

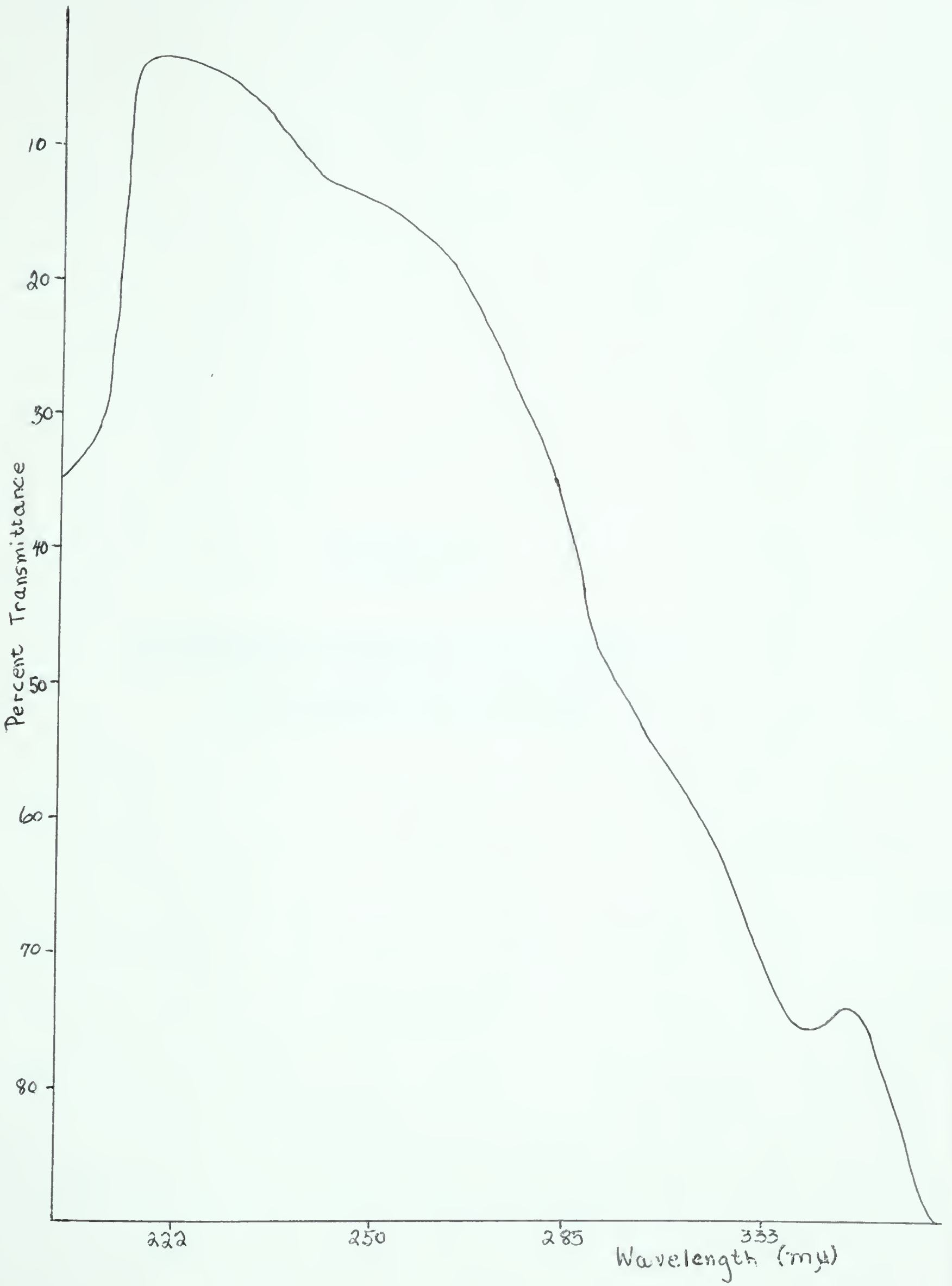

10
20
30
40
50
60
70
80
Percent Transmittance
222
250
285
333
Wavelength (mμ)

FIGURE VII

UV and Visible Spectra of the Pigment of

M. violagabriellae in H_3PO_4

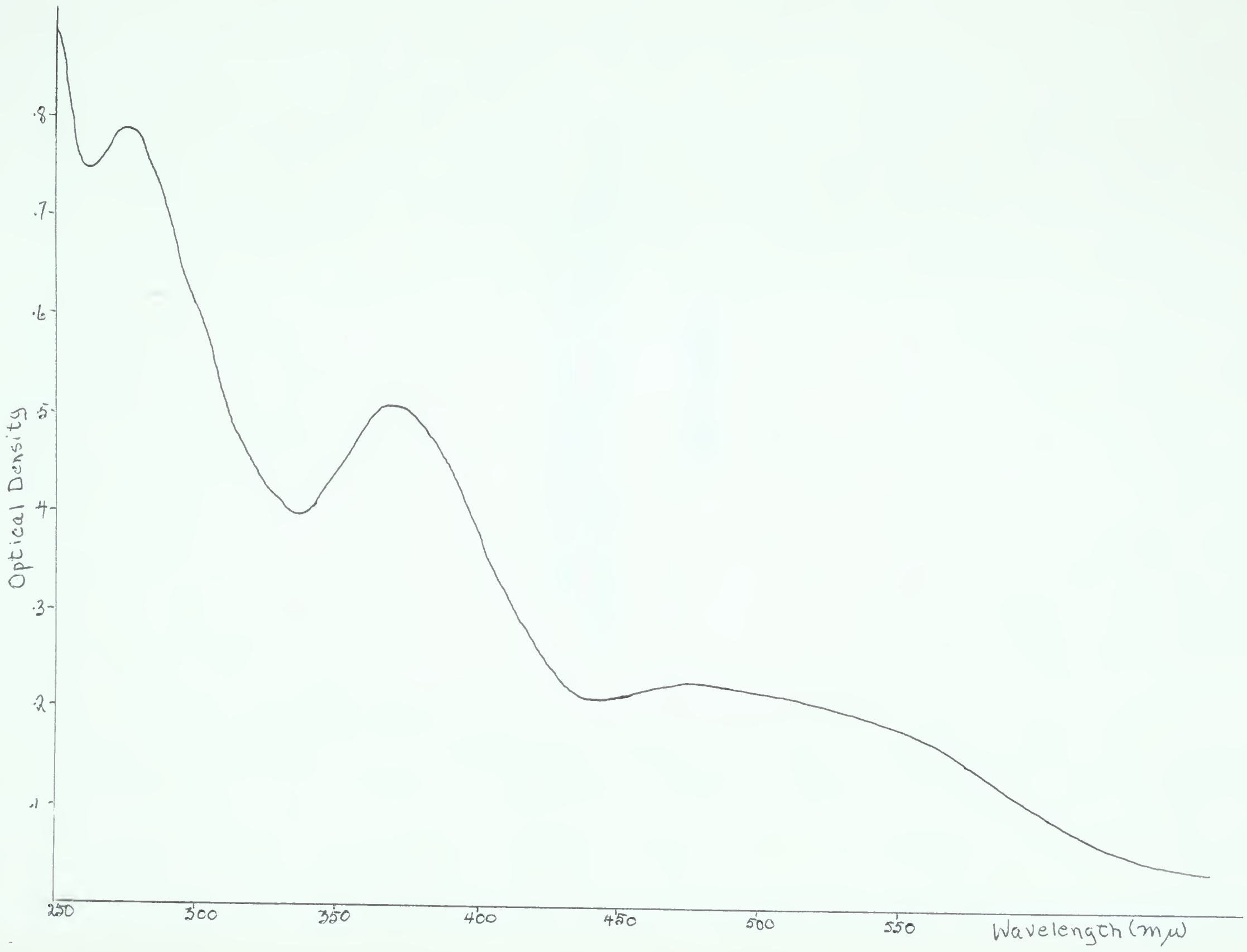
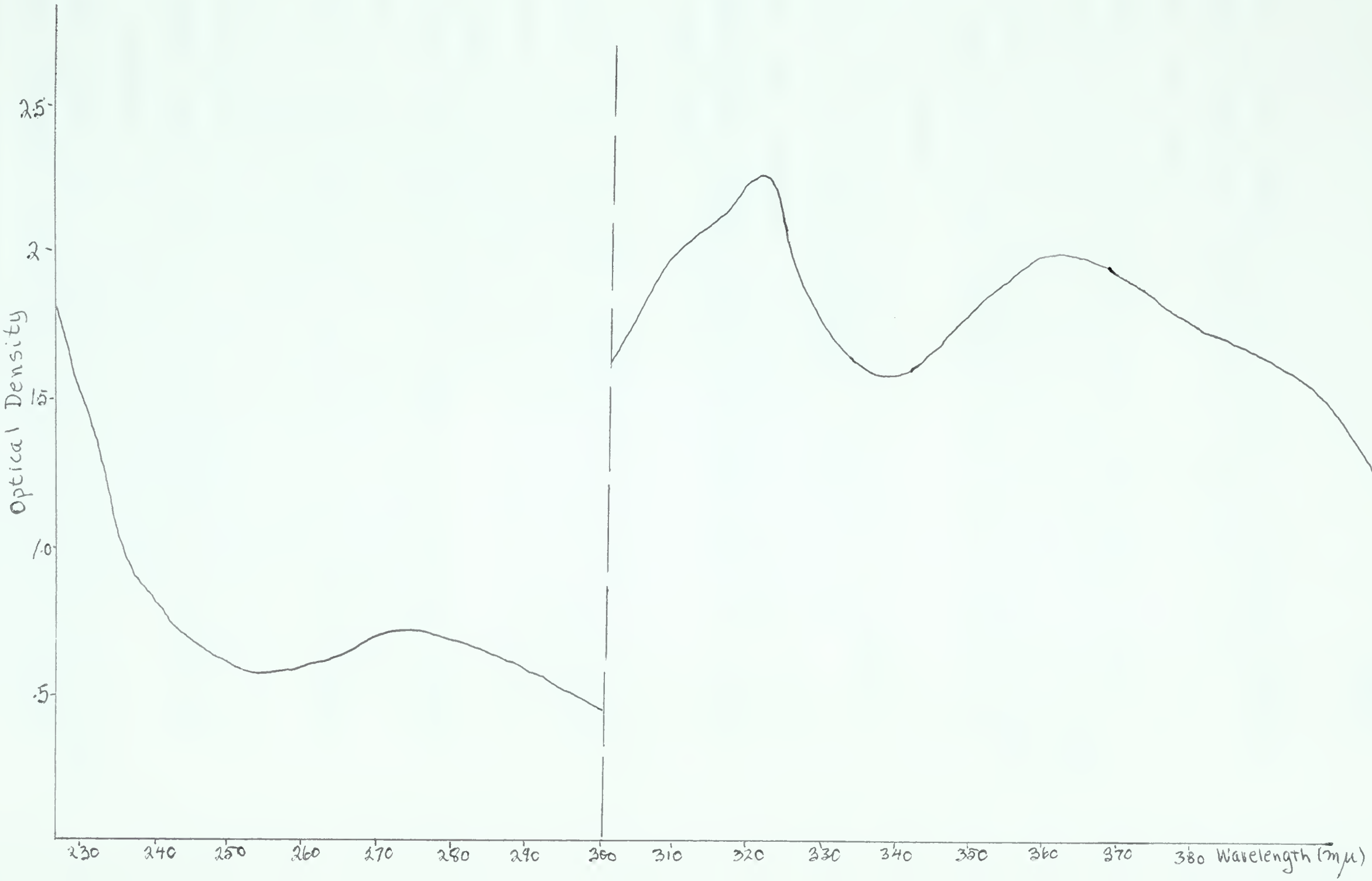

Optical Density
.8
.7
.6
.5
.4
.3
.2
.1
250
300
350
400
450
500
550
Wavelength (mμ)

FIGURE VIII

UV and Visible Spectra of the Pigment of M. violagabriellae in HCl


Optical Density
2.5
2.
1.5
1.0
.5
230
240
250
260
270
280
290
300
310
320
330
340
350
360
370
380 Wavelength (mμ)

solution. This change in color could be correlated with a decrease in ability to precipitate the pigment from solution using acid.

After 18 to 24 hours in alkali, the color of the precipitated pigment was a light pink; after 42 hours, when the alkaline solution was colorless, the precipitate formed on the addition of acid was white (hereafter referred to as 'white' pigment).

The loss of yellow color of the pigment in alkali was found to be oxygen dependent. Solutions left standing under anaerobic conditions did not change color, but remained an intense yellow and could still be precipitated with acid.

In addition, the more times the alkali-acid conversion was carried out, the more difficult it was to precipitate the pigment by lowering the pH. The pigment was at all times readily soluble in alkali.

Figure IX (page 65) illustrates the spectral changes of alkaline solution of the pigment at different time intervals. The broad peak between 227 and 246 mu remains stable, but peaks at 284 and 413 mu are lost.

To determine what, if any, components were lost from the pigment during alkaline decomposition, the 'red' and 'white' pigments were compared in several ways.

The red and white pigments were hydrolized and examined for amino acid and carbohydrate composition, as described previously. No differences between the two were found.

FIGURE IX

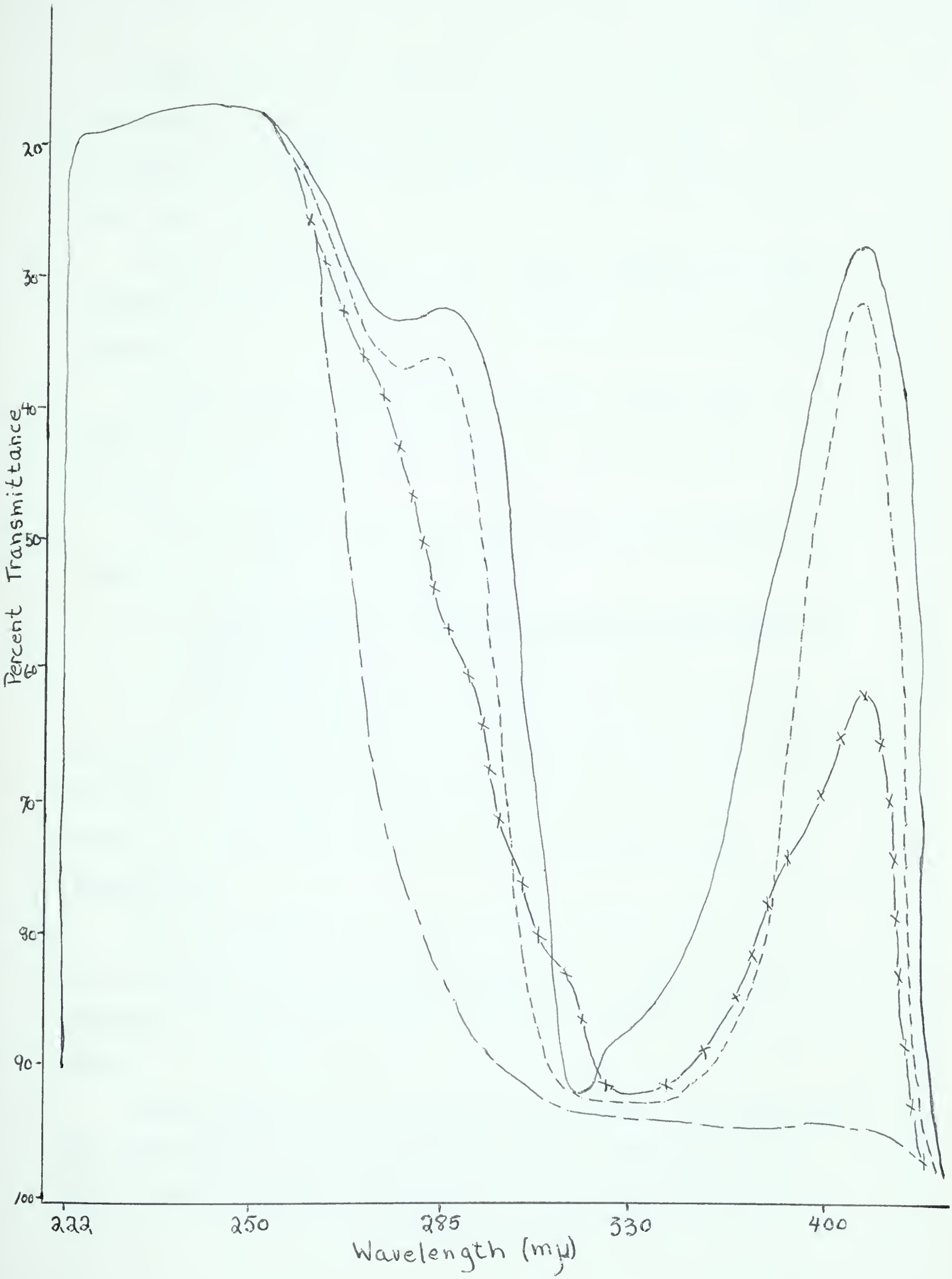
Illustration of the Spectral Changes of the Pigment of M. violagabriellae in Alkali

——— 0 Time

------ 1 Hour

— x — 3 Hours

— - — 3 Weeks


Percent Transmittance
20
30
40
50
60
70
80
90
100
222
250
285
330
400
Wavelength (mμ)

After drying the 'white' pigment in a vacuum desiccator, it became a light brown color. The solubility characteristics were identical to those of the 'red' pigment.

Absorption spectrum of the 'white' pigment in N KOH revealed maxima at 222 mu and a shoulder at 242 mu (Figure X) (page 67).

IR spectrum of the 'white' pigment (Figure XI) (page 68) was similar to that of the 'red' pigment (Figure V) (page 60).

The data above indicates that the red and white pigments are very closely related.

12. Precipitation of Iron from Alkaline Solutions of the Pigment

Coincident with a loss of color, decreasing ability to precipitate the pigment by dropping the pH, and loss of color of the acid precipitate from an alkaline solution, there was a formation of an orange-brown precipitate.

The precipitate was found to be insoluble in 95 per cent ethanol, petroleum ether andwater. It was, however, soluble in concentrated HCl, in which it gave a yellow color.

After partial neutralization with water, the material was spotted on chromatograms and run in n-butanol-acetic acid-water. Location reagents ninhydrin and aniline-

FIGURE X

UV and Visible Spectra of 'White' Pigment

from M. violagabriellae

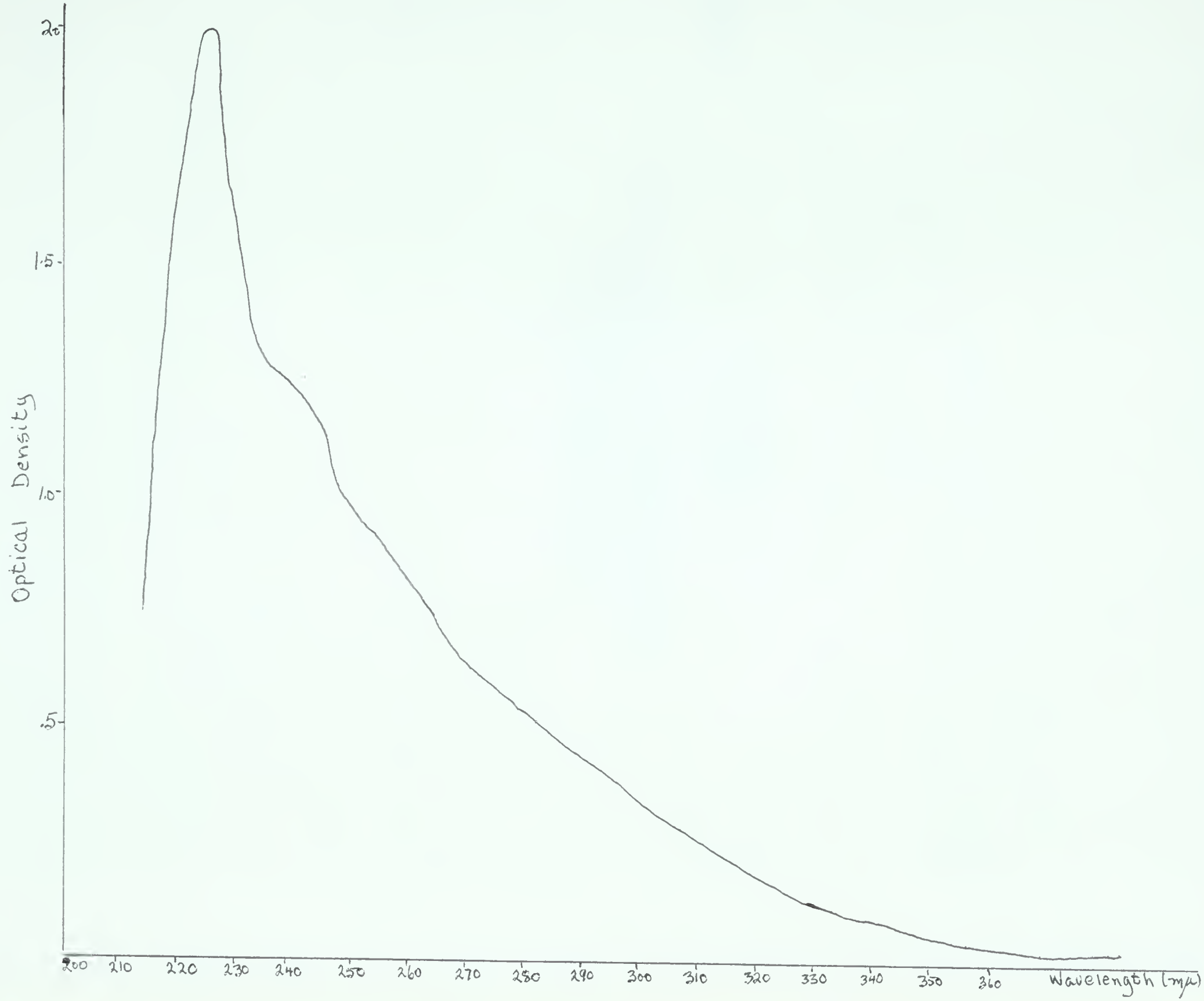
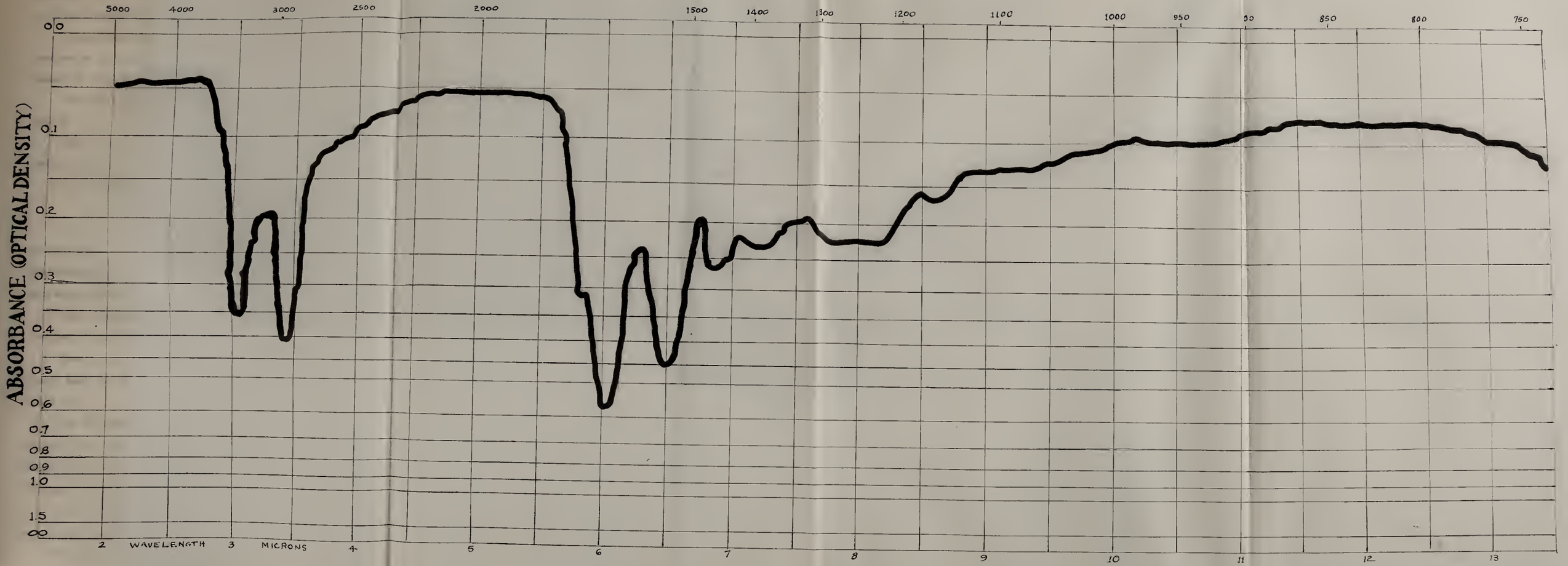
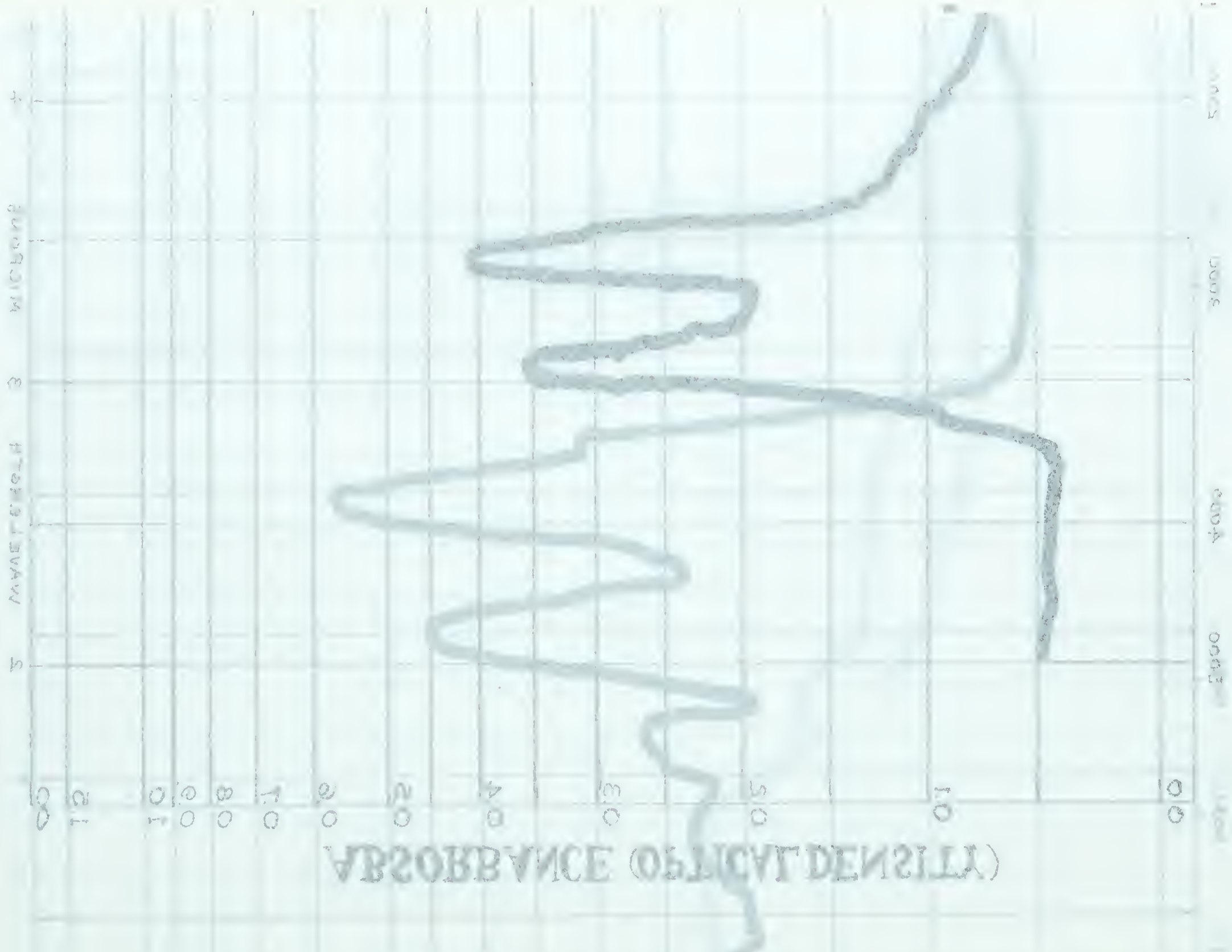

Optical Density
2.0
1.0
.5
200
210
220
230
240
250
260
270
280
290
300
310
320
330
340
350
360
Wavelength (mμ)

FIGURE XI

IR Spectrum of the 'White' Pigment

from M. violagabriellae


5000
4000
3000
2500
2000
1500
1400
1300
1200
1100
1000
950
900
850
800
750
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
1.5
∞
ABSORBANCE (OPTICAL DENSITY)
2
WAVELENGTH
3
MICRONS
4
5
6
7
8
9
10
11
12
13


WAVELENGTH
ABSORBANCE (OPTICAL DENSITY)

phthalic acid did not reveal the presence of amino acid or sugar components.

Spectrophotometric study of the precipitate revealed maxima at 212 and 240 mu (Figure XII) (page 70), but no peaks in the IR spectrum.

The melting point of the precipitate was above 300 C.

Spot tests performed on the precipitate (Feigl, 1946) demonstrated the presence of ferric iron.

13. Isolation of Pigment from Cells Grown in Absence of Iron

Cells grown on media containing no iron were extracted as for normal pigmenting cultures involving alkali-acid purification. Upon the addition of acid to the alkali extracted cells, a white precipitate was formed.

This 'pigment' was soluble in alkali, but not in acetone, chloroform or water.

Spectroscopic analyses of this pigment revealed a sharp peak at 225 mu with a shoulder at 242 mu (Figure XIII) (page 71).

The IR spectrum was similar to that of the red pigment, but showed no peaks from 1050 to 700 cm^{-1}. Maxima at 1217, 1155 and 1068 cm^{-1} were greatly reduced (Figure XIV) (page 72).

Unlike the red pigment, it could not reduce ammoniacal silver nitrate.

Preparation of 22 and 1-hour hydrolyzates

FIGURE XII

UV Spectrum of the Precipitate from an Alkaline Solution of the Pigment of M. violagabriellae

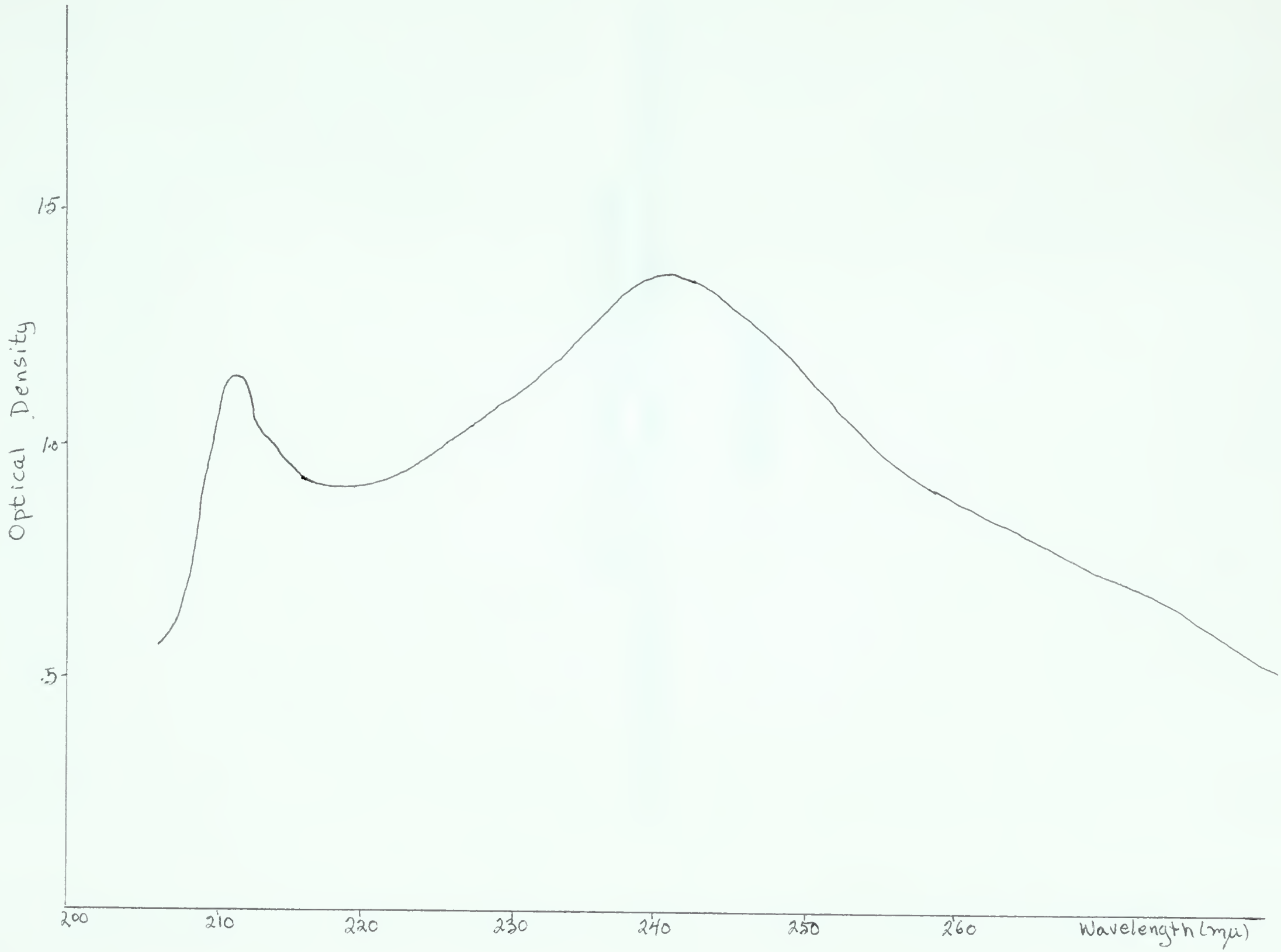

Optical Density
1.5
1.0
.5
200
210
220
230
240
250
260
Wavelength (mμ)

FIGURE XIII

UV and Visible Spectra of Pigment of M. violagabriellae Grown in the Absence of Iron

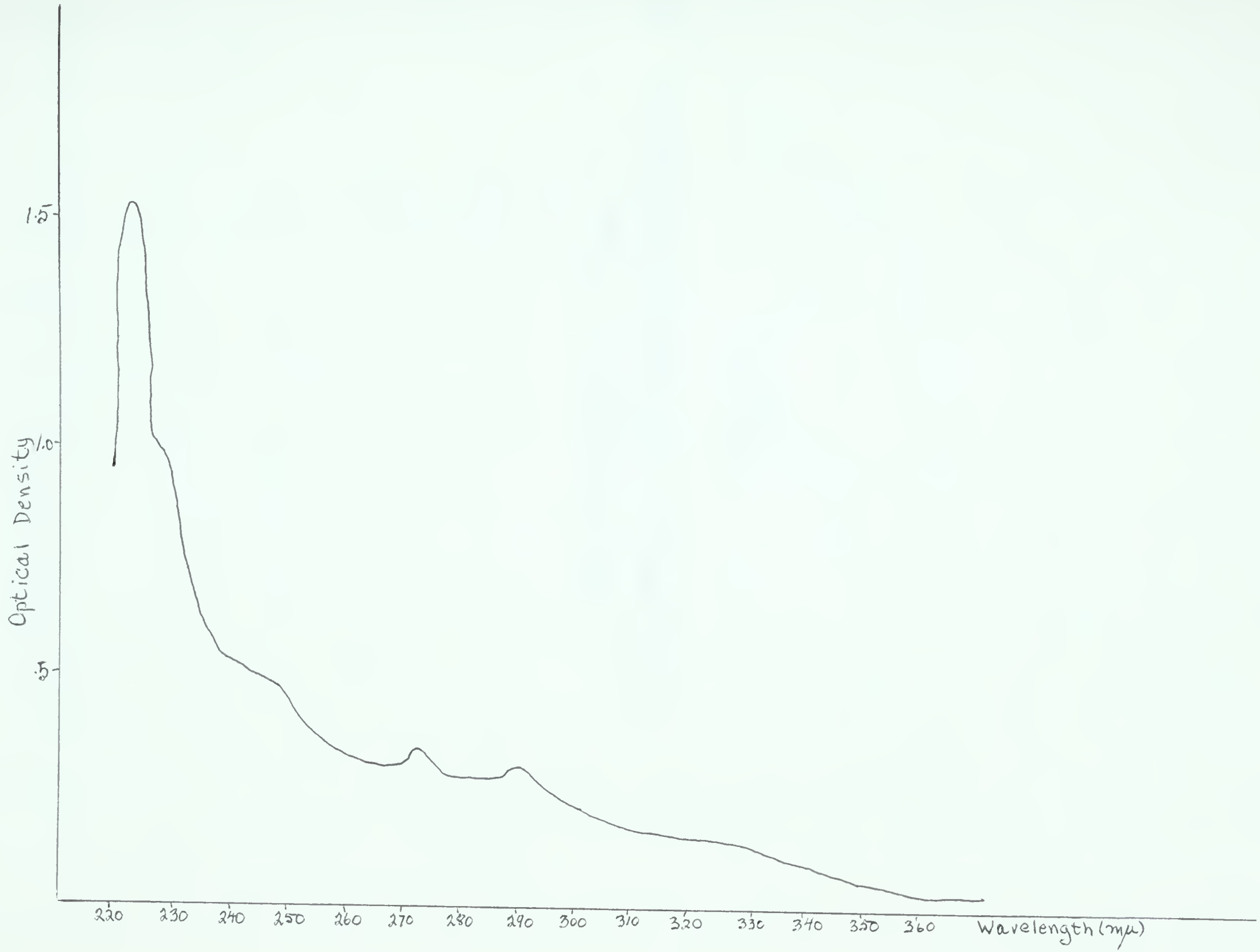
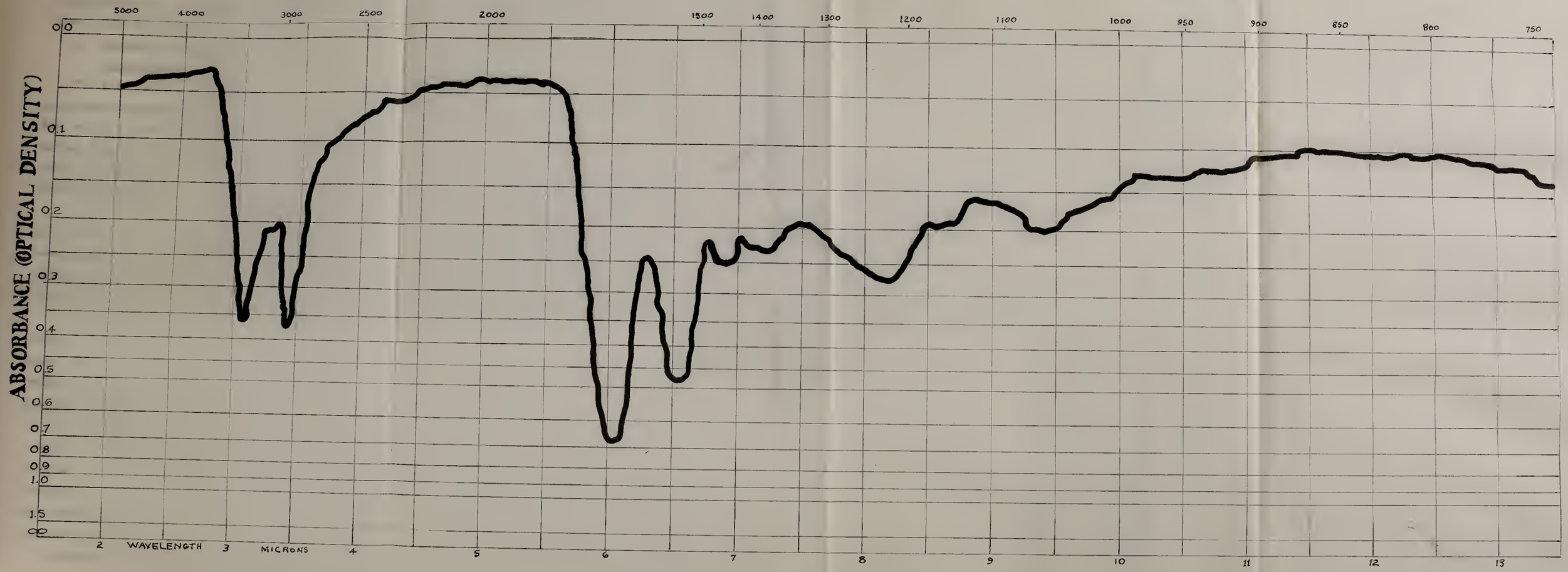
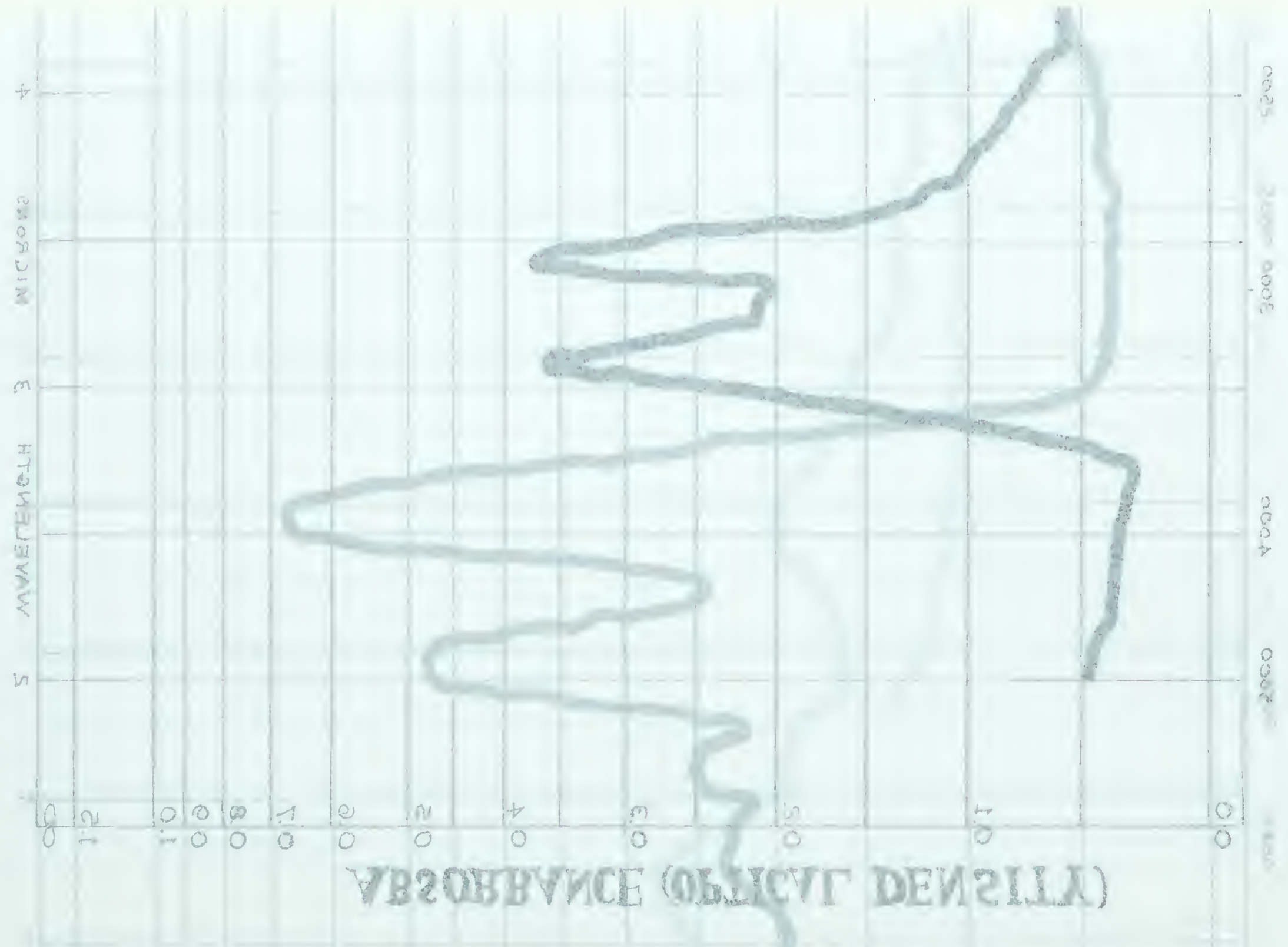

Optical Density
1.5
1.0
.5
220
230
240
250
260
270
280
290
300
310
320
330
340
350
360
Wavelength (mμ)

FIGURE XIV

IR Spectrum of Pigment of M. violagabriellae
Grown in the Absence of Iron


5000
4000
3000
2500
2000
1500
1400
1300
1200
1100
1000
950
900
850
800
750
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
1.5
∞
ABSORBANCE (OPTICAL DENSITY)
2
WAVELENGTH
3
MICRONS
4
5
6
7
8
9
10
11
12
13


ABSORBANCE (OPTICAL DENSITY)
WAVELENGTH
ANTHRACIN

chromatographically revealed the same amino acids and sugar as the red pigment.

14. Studies on the Effect of Various Compounds on Pigmentation

Table X (page 74) shows the effect of 3 metabolic inhibitors on pigmentation. Concentrations of 10^{-4} M NaN_3 and 10^{-3} M KCN inhibited pigmentation to a marked degree, but did not inhibit growth. NaF had no inhibiting effect on pigmentation and was actually stimulatory in the higher concentrations.

10^{-2} M p-cresol, 10^{-2} M o-cresol, 10^{-3} M m-cresol, 10^{-3} M ascorbic acid, 10^{-2} M glutathione, 10^{-2} M p-benzyloxyphenol and 1:100,000 methylene blue were inhibitory to pigmentation but not to growth (Table XI) (pages 75, 76).

By inoculating plates containing the following compounds in concentrations between 0.01 mg/ml and 0.8 mg/ml, no visible effect on pigmentation could be detected: cysteine, phenol, pyrocatechol, p-phenylene-diamine, hydroquinone and aniline.

When pigmentation was inhibited by using 0.01 M p-cresol, reversal of inhibition could not be accomplished using aromatic intermediates such as tyrosine in concentrations of 1 M to 1 x 10^{-5} M, phenylacetic acid in concentrations varying from 1 M to 1 x 10^{-4} M, or shikimic acid in concentrations varying from 1 x 10^{-2} M to 1 x 10^{-5} M.

Although cells grown in shake cultures in the presence

TABLE X

Effect of Respiratory Poisons on Pigmentation in *M. violagabriellae*

Compound	Growth Response (O.D. at 600 mu)	Degree of Pigmentation (O.D. of alkali extract of culture at 413 mu
.01 M NaN_3	-	-
.001 M NaN_3	.355	.076
.0001 M NaN_3	.48	.137
.00001 M NaN_3	.49	.315
Control (no NaN_3)	.48	.310
.1 M NaF	.135	.458
.01 M NaF	.44	.349
.001 M NaF	.525	.315
.0001 M NaF	.51	.325
.00001 M NaF	.52	.325
Control (no NaF)	.51	.329
.1 M KCN	-	-
.01 M KCN	.10	.074
.001 M KCN	.51	.287
.0001 M KCN	.60	.342
.00001 M KCN	.57	.369
Control (No KCN)	.53	.342

TABLE XI

Effect of Various Compounds on Pigmentation in M. violagabriellae

Compound	Growth Response (O.D. at 600 mu)	Degree of Pigmentation (O.D. of alkali extract of culture at 413 mu
.2 M p-cresol	-	-
.1 M p-cresol	.355	0
.01 M p-cresol	.585	.100
.001 M p-cresol	.565	.285
.0001 M p-cresol	.542	.330
Control (no p-cresol)	.542	.350
.1 M o-cresol	-	-
.01 M o-cresol	.61	.037
.001 M o-cresol	.66	.187
.0001 M o-cresol	.65	.251
Control (no o-cresol)	.63	.406
.1 M m-cresol	-	-
.01 M m-cresol	.46	.035
.001 M m-cresol	.65	.035
.0001 M m-cresol	.65	.225
Control (no m-cresol)	.63	.406
.1 M ascorbic acid	.40	0
.01 M ascorbic acid	.40	0
.001 M ascorbic acid	.49	.034
.0001 M ascorbic acid	.51	.237
.00001 M ascorbic acid	.48	.320
Control (no ascorbic acid)	.49	.316

(continued on page 76)

TABLE XI (continued)

Compound	Growth Response (O.D. at 600 mu)	Degree of Pigmentation (O.D. of alkali extract of culture at 413 mu
.01 M glutathione	.385	.093
.001 M glutathione	.370	.156
.0001 M glutathione	.370	.176
.0001 M glutathione	.390	.195
Control (no glutathione)	.40	.258
.01 M p-benzyloxyphenol	.47	.027
.001 M p-benzyloxyphenol	.54	.328
.0001 M p-benzyloxyphenol	.54	.300
Control (no p-benzyl-oxyphenol)	.55	.355
1:100,000 methylene blue	.285	.028
1:500,000 methylene blue	.335	.221
Control (no methylene blue)	.36	.235

of dihydroxyphenylalanine appeared to produce a darker pigment, it was not known to what extent this was contributed to by the presence of dopa-melanin, produced by auto-oxidation.

Sonically disrupted pigmenting cells (18 hours incubation), when tested by the method of Arnow (1937), failed to show the presence of dopa.

15. Effect of Inhibition by p-cresol on Pigmentation

Cells grown in basal medium containing 0.01 M p-cresol produced a brown compound, after 36 hours of incubation, which was soluble in the basal medium. Uninoculated controls did not show this brown compound.

Table XII (page 78) shows the solubilities of this compound. Of the solvents tried, the compound was soluble only in water.

Figure XV (page 79) shows the UV and visible absorption spectra of this compound purified by paper chromatography using the n-butanol-acetic acid-water solvent system.

The compound is characterized by non-specific absorption in the region 200-600 mu. It has however maxima at 193, 191 and 189 mu.

The IR spectrum of the pigment (Figure XVI) (page 80) shows maxima at 3350, 2940, 1580, 1420 cm^{-1}, a broad peak between 1070 and 1150, 1030, and 790 cm^{-1}.

16. Melanins from Other Microorganisms

In order to compare the melanin-like pigment of M. violagabriellae with that of other 'melanin' producing

TABLE XII

Solubility Characteristics of 'Melanin' Pigments of Pseudomonas and Pigment in p-cresol Inhibited Cultures of M. violagabriellae

Solvent	P. aeruginosa Strain MS	P. aeruginosa Strain M 117	Pigment from p-cresol inhibited M. violagabriellae cultures
Ethylacetate	NS*	NS	-
Benzene	NS	NS	NS
95% ethanol	VSS	NS	NS
Chloroform	NS	NS	NS
n-butanol	NS	NS	NS
Isopropanol	NS	NS	NS
Pyridine	NS	SS	NS
Water	S	S	S
2-chloroethanol	VSS	SS	NS
Acetone	NS	NS	NS
Petroleum ether	NS	NS	NS
Methanol	SS	SS	NS
Heptane	NS	NS	NS
N KOH**	S	S	S

* NS = not soluble
VSS = very slightly soluble
SS = slightly soluble
S = soluble

** non-precipitable with acid

FIGURE XV

UV and Visible Spectra of 'Pigment'
from p-cresol Inhibited Cultures of M. violagabriellae

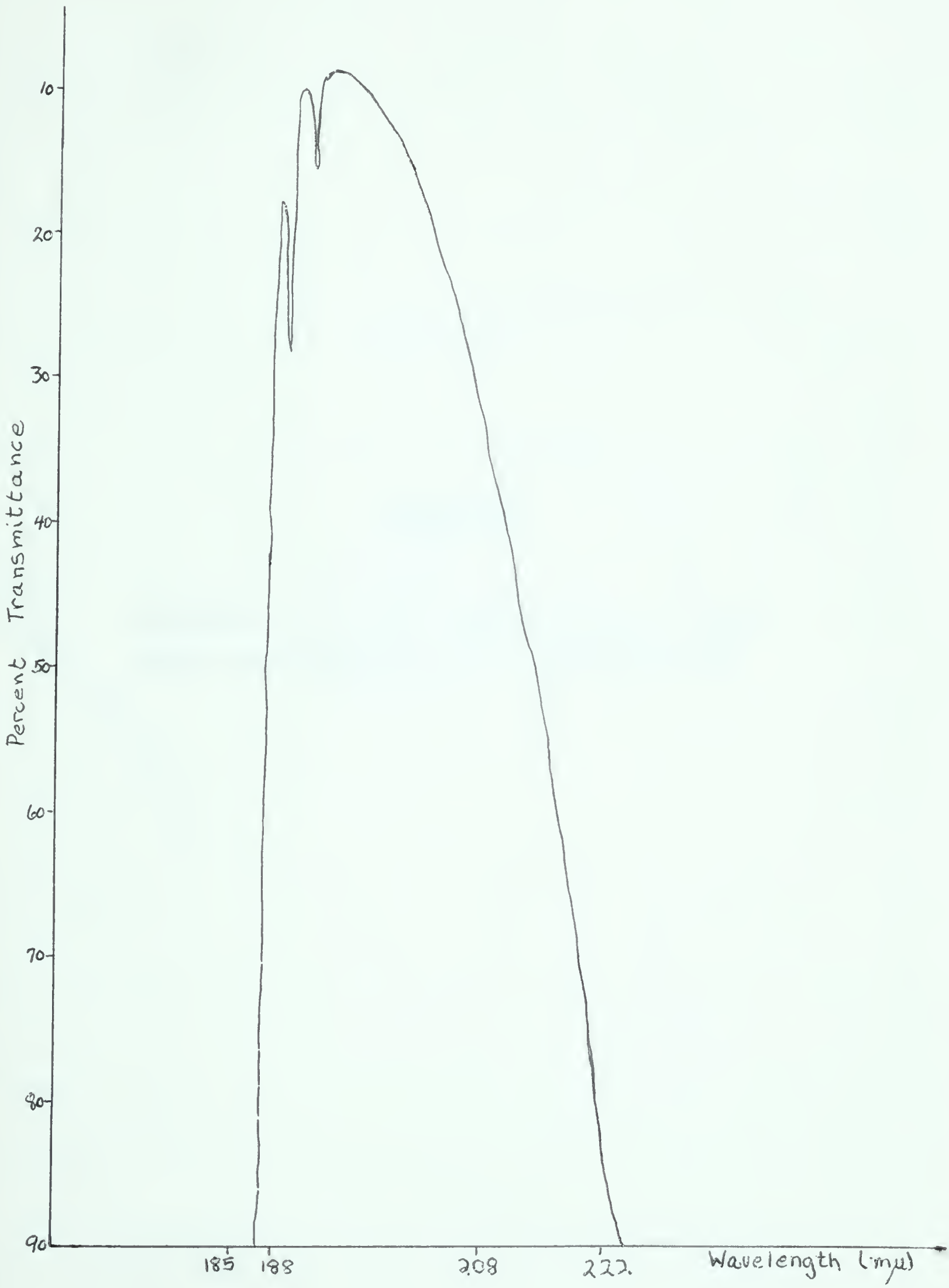
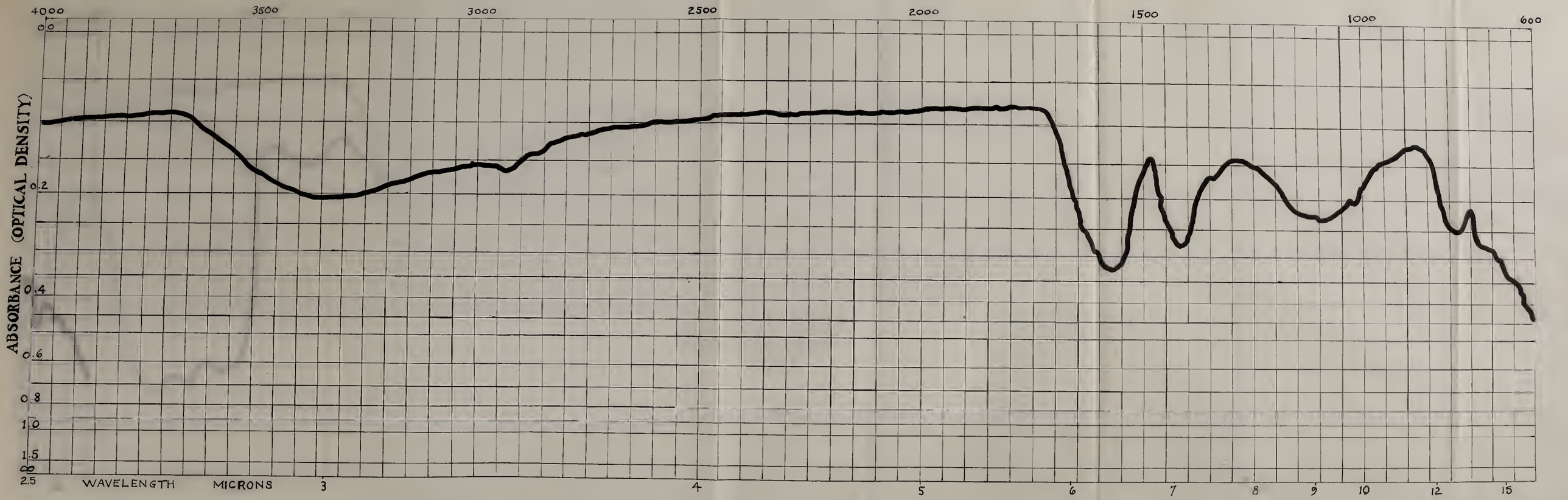
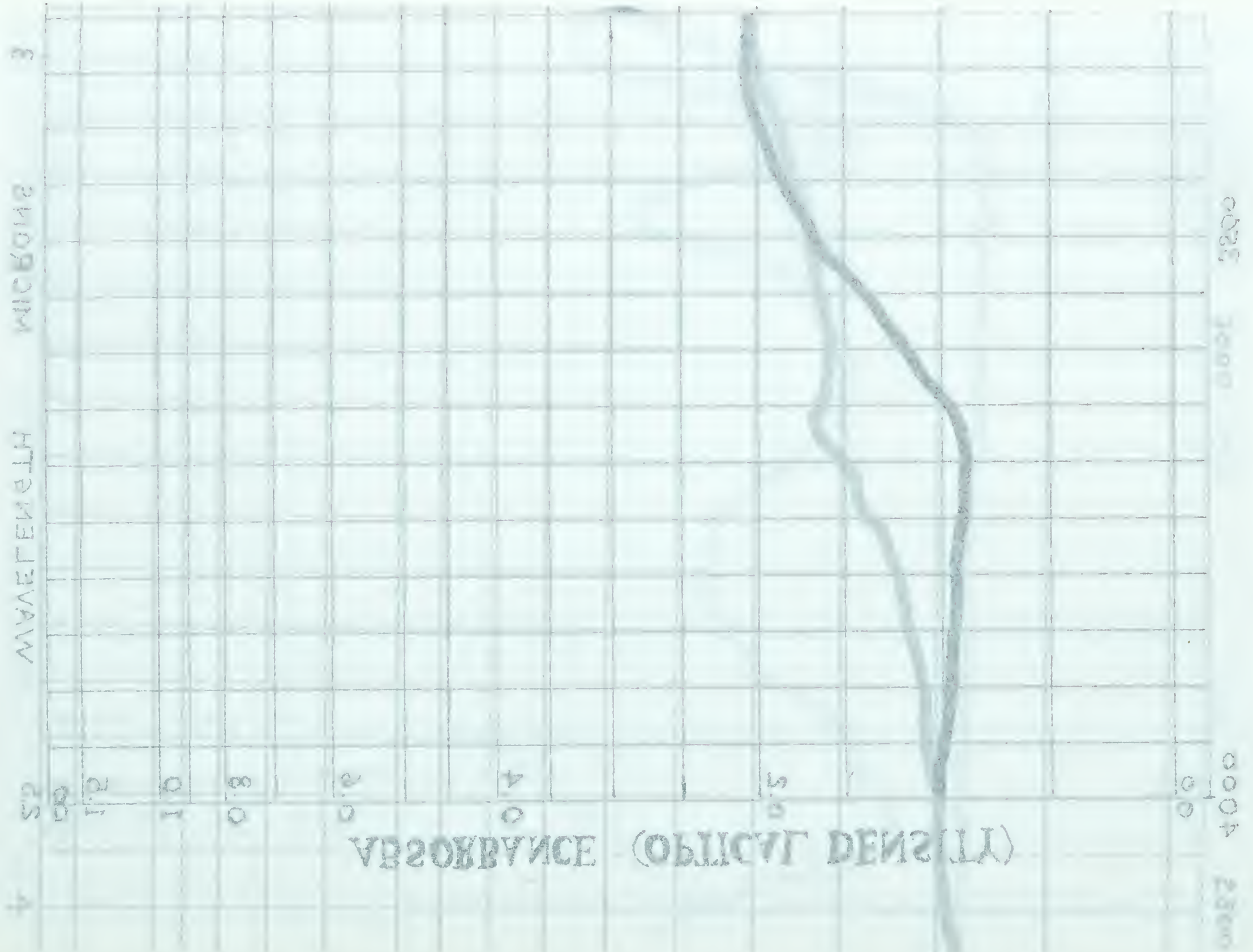

Percent Transmittance
10
20
30
40
50
60
70
80
90
185
188
208
222
Wavelength (mμ)

FIGURE XVI

IR Spectrum of the 'Pigment' from p-cresol Inhibited Cultures of M. violagabriellae


4000
3500
3000
2500
2000
1500
1000
600
ABSORBANCE (OPTICAL DENSITY)
0.0
0.2
0.4
0.6
0.8
1.0
1.5
2.0
2.5
WAVELENGTH MICRONS
3
4
5
6
7
8
9
10
12
15


WAVELENGTH MICRONS
ABSORBANCE (OPTICAL DENSITY)

bacteria, the pigments produced by two Pseudomonas species were studied.

The M 117 strain of Pseudomonas aeruginosa (Inoue, 1961, kindly supplied by Dr. Inoue) and the MS strain of P. aeruginosa (Gessard, 1901, kindly supplied by Dr. Buttiaux) were grown on Trypticase soy agar.

Both pigments diffused into the agar medium and could be extracted with water. Table XII (page 78) includes the solubility characteristics of both pigments.

Pigment of both strains was characterized by non-specific absorption. The MS strain showed absorption maxima identical to that for the brown compound of p-cresol inhibited cultures of M. violagabriellae, with maxima at 193, 191 and 189 mu (Figure XV) (page 79).

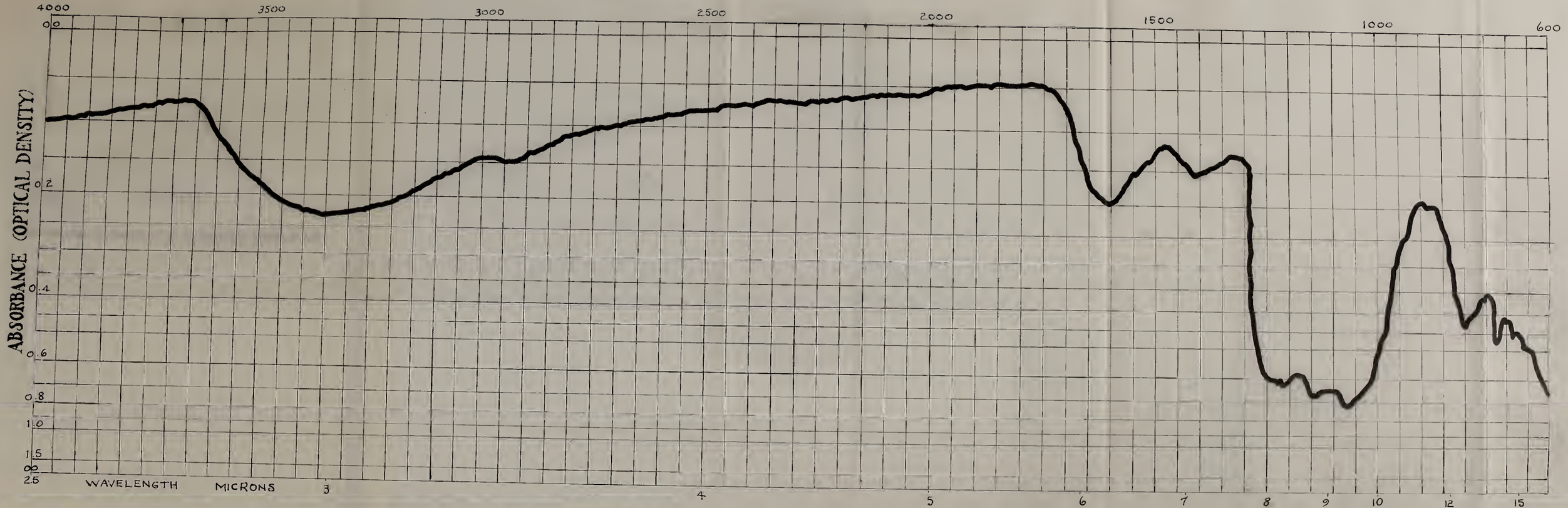
The M 117 pigment was not examined at the wavelengths below 200 mu.

The IR spectrum of the purified pigment from the MS strain showed absorption maxima at 3350, 2950, 1600, 1420, 1220, 1140, 1070, 790 and 730 cm^{-1} (Figure XVII) (page 82).

The IR spectrum of the M 117 pigment, prepared in the same way, showed absorption maxima at 3350, 2930, a broad peak at 1650, 1400, 1230, 1140, 1070, 780 and 730 cm^{-1} (Figure XVIII) (page 83).

FIGURE XVII

IR Spectrum of Pigment of P. aeruginosa, Strain MS


4000
3500
3000
2500
2000
1500
1000
600
0.0
0.2
0.4
0.6
0.8
1.0
1.5
2.0
2.5
ABSORBANCE (OPTICAL DENSITY)
WAVELENGTH MICRONS
3
4
5
6
7
8
9
10
12
15

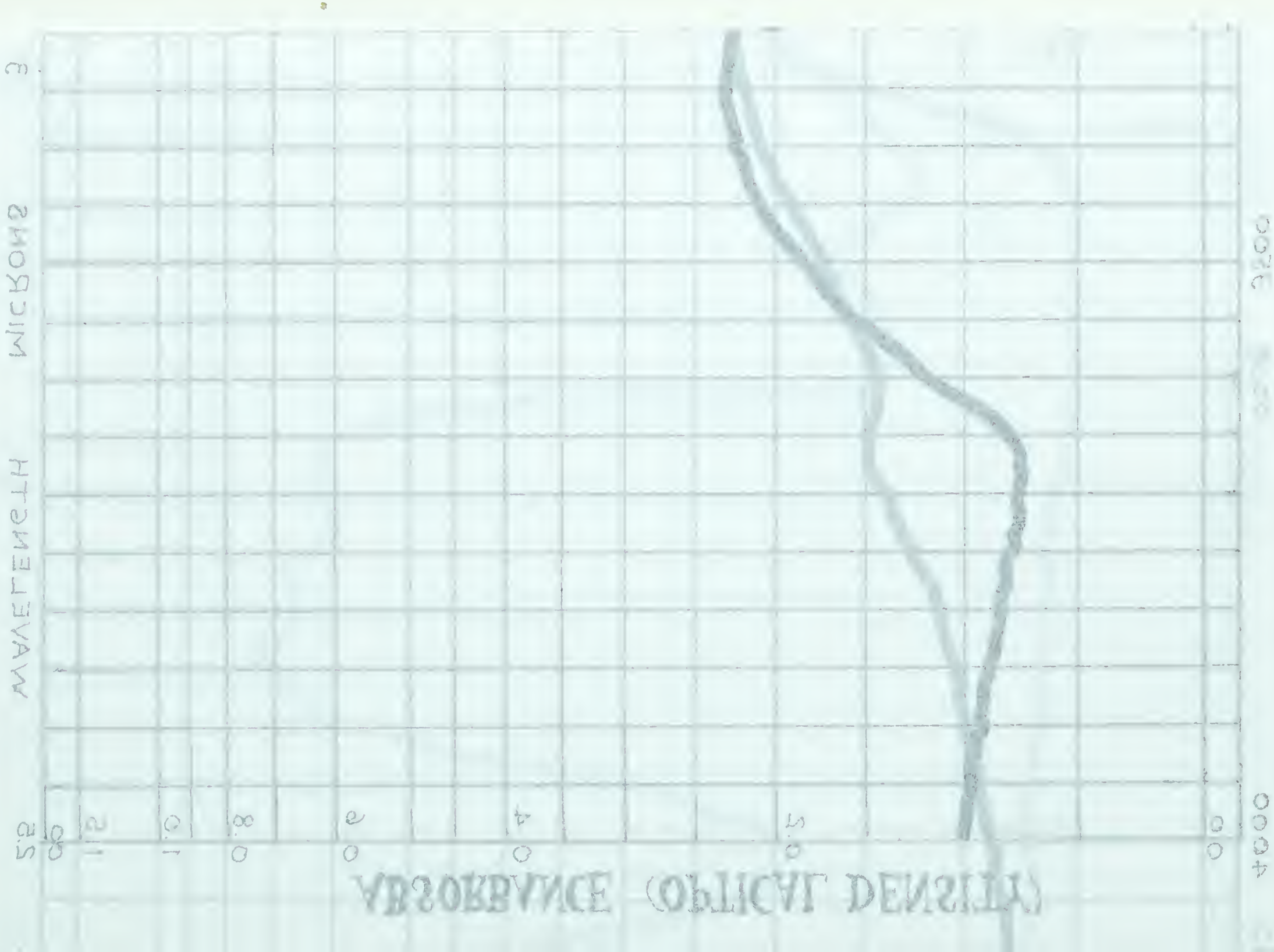
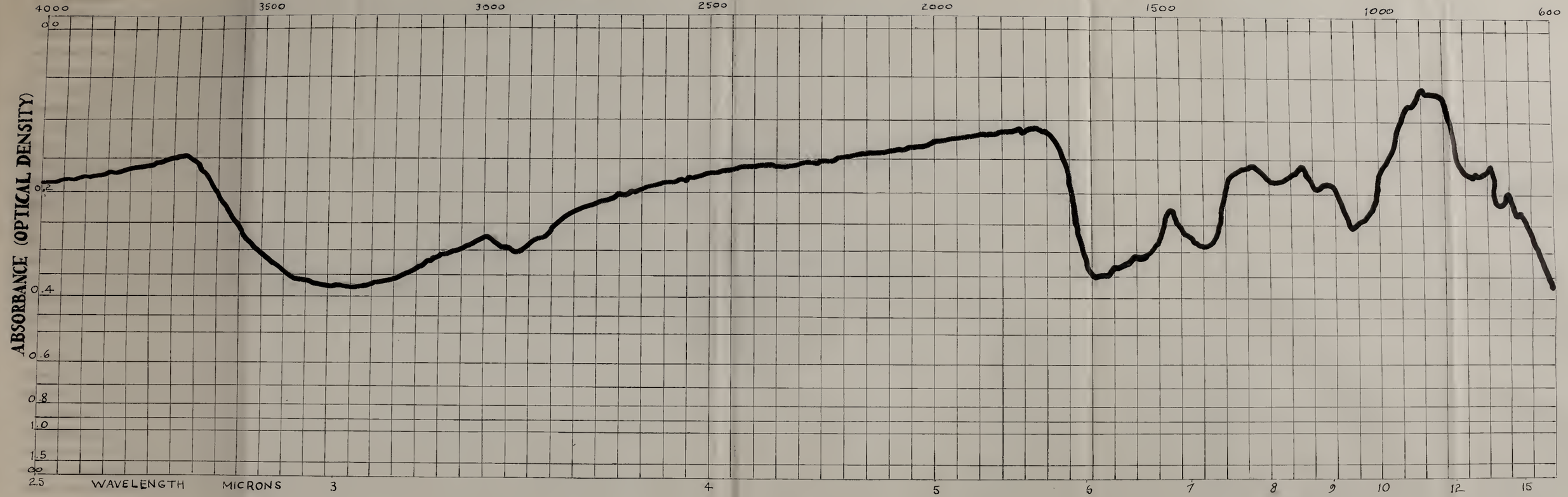

WAVELENGTH
ABSORBANCE (OPTICAL DENSITY)

FIGURE XVIII

IR Spectrum of Pigment of P. aeruginosa, Strain M 117


4000
3500
3000
2500
2000
1500
1000
600
0.0
0.2
0.4
0.6
0.8
1.0
1.5
2.5
ABSORBANCE (OPTICAL DENSITY)
WAVELENGTH MICRONS
3
4
5
6
7
8
9
10
12
15


WAVELENGTH (MICRONS)
ABSORBANCE (OPTICAL DENSITY)

D. Discussion

The reduction of ammoniacal silver nitrate, characteristic of the pigment isolated, is used as a test to identify melanins and is used to a large extent in histochemical studies (Pearse, 1960; Young, 1961).

The pigment isolated is unlike melanins in that bleaching with oxidizing agents could not be accomplished.

Melanins are characterized by their insolubility in organic solvents and in neutral or acidic aqueous solutions, and their solubility in aqueous alkali and concentrated sulphuric acid. Dilution of the sulphuric acid system reprecipitates the pigment, as does the neutralization of the alkaline solution (Fox, 1953).

These solubility characteristics are identical to those for the pigment of M. violagabriellae.

Lloyd and Johnston (1932) reported that a bacillus isolated from sea water produced a dark brown pigment which was soluble in NaOH and could be reprecipitated by neutralization of the solution.

Mencher and Heim (1962) found that the melanin pigment of Streptomyces lavendulae was soluble in M-NaOH and could be precipitated by acid.

Baichwal and Walker (1960) extracted pigments of Trichophyton rubrum using 5 per cent NaOH. Schaeffer (1953) used 0.5 N NaOH to extract the black pigment of a mutant of Neurospora crassa. In the latter case, the pigment could be precipitated by acid.

In non-microbial systems, melanins have also been extracted using aqueous alkali. Gortner (1911) could extract the pigment in black wool by dilute alkali, as could Serra (1946) from the hair of rabbits.

There are, however, exceptions to these solubility characteristics. Greenstein, Turner and Jenrette (1940) found that melanin from a mouse melanoma could be dissolved in neutral buffers and in water.

The melanin pigments of P. aeruginosa strain MS (Gessard, 1901) and Strain M 117 (Inoue, 1961) studied herein were both soluble in water and in this respect differ from the pigment of M. violagabriellae.

Lea (1945) found that ink sac melanin of Sepia officinalis, melanoma melanin, and melanin obtained by the action of tyrosinase on tyrosine were soluble in cold ethylene chlorohydrin. The substance remaining after evaporation of the solvent was indistinguishable from the original melanin.

Millott and Jacobson (1952) found that the melanin in the sea-urchin Diadema antillarum was not soluble in ethylene chlorohydrin. Nor could Fontaine (1962) dissolve the melanin pigment of Ophiocomina nigra (Abildgaard) in ethylene chlorohydrin.

The pigment of M. violagabriellae was slightly soluble in ethylene chlorohydrin and pyridine, but only after alkali-acid treatment. The solvents when evaporated left a yellow colored residue, indicating that the native state of the

pigment had been changed.

These observations would suggest that alkaline hydrolysis had taken place even though the pigment was exposed for only short periods of time.

The pigments from the 2 strains of Pseudomonas aeruginosa were soluble in ethylene chlorohydrin to only a slight extent.

M. violagabriellae pigment could be extracted with butyl alcohol from aqueous perchloric acid solution, but not from aqueous alkaline solutions. Since the dissolution of the pigment in butanol was possible only after 72 hours in acid, hydrolysis must have occurred and the pigment extracted was not identical to the native pigment.

Similarly, Arnow (1938) found that the acid-soluble pigment of human red hair could be extracted from acid solutions with butanol.

Unlike some of the microbial melanins studied by Skinner (1939), M. violagabriellae pigment did not change from red to black when extracted with alkali, and the red color of the pigment is undoubtedly the color of the terminal product of oxidation.

The solubility characteristics of the isolated pigment, in addition to being similar to those of melanins, are also similar to those reported for the red, iron-dependent pigment, pulcherrimin, isolated from Candida pulcherrima (Kluyver, van der Walt and van Triet, 1953).

Melanins isolated from natural sources are often conjugate or combined pigments (Mason, 1948); that is, the melanin can be associated with other components. Examples are melanoproteins, melanopolypeptides and melanolipoids.

The pigment of M. violagabriellae has associated with it a peptide and pentose component. The peptide component contains 6 different amino acids and since analysis revealed only one N terminal amino acid, the peptide is probably attached to the chromophore in only one place on the molecule.

Greenstein (1948) isolated a melanin-containing protein from a melanoma; Gortner (1911) found that the melanin in black wool was a melanoprotein; Serra (1946) extracted melanins from the hair of black, brown and yellow rabbits which he described as melanoproteins; Arnow (1938) found that acid-soluble pigment from red human hair gave positive tests for protein.

Although attempts to remove the protein moiety from the pigment of M. violagabriellae were unsuccessful, other workers could separate these 2 components. Serra (1946) obtained hair melanin free of protein by hydrolysis. Others such as Bonner and Duncan (1962) and Greenstein et al. (1941) could remove the protein by enzymic hydrolysis.

The dependence of pigmentation on iron was demonstrated earlier in Chapter I. Iron was also found to be incorporated

into the pigment, but could be removed by alkali treatment.

The removal of iron from the pigment did not affect the color of the pigment, and iron is therefore not necessary for the color of the pigment.

Furthermore, a white pigment was isolated from cells grown in the absence of iron, which appeared to differ from the red pigment only in its color and inability to reduce ammoniacal silver nitrate.

The association of iron with melanins has been reported by many workers. Rotman and Flesch (1943) isolated an iron pigment from human red hair which contained 10.21 per cent ash, high in iron content.

Eckardt, Stolzar, Adam and Johnson (1943) found abnormal cations of zinc, copper and iron in ashed corneas from two cases of Wilson's disease (deposition of green-brown pigment in cornea).

Laxer and Whewell (1954) found that melanin granules isolated from pigmented mammalian hairs contained 0.1 to 0.5 per cent iron.

Red and buff feathers yielded a component of red melanin which was iron-containing (Nickerson, 1946). Schaaf (1929) also describes an iron-containing melanin.

Thus there is much evidence which suggests that a number of metals combine with and perhaps influence the color of natural melanin complexes.

Bowness and Morton (1953) carried out experiments on the effects of various metals on the rate of oxidation of

dopa to melanin by H_2O_2. It was found that Cu, Co, Fe++ and Fe+++ accelerated melanin formation under these conditions.

Flesch (1949) demonstrated the autoxidation of dopa in vitro was more strongly catalyzed by cupric ions than by any of the other heavy metallic salts, but that Fe did stimulate autoxidation.

Robert and Zurcher (1950) could increase dopa-melanin in vitro by the addition of Ag, Cu, Mn, Au, Fe or Cu. Intracutaneous injections of solutions of Fe, Cu, Co, Ni and As salts produced local pigmentation and accelerated growth of hair in experimental animals.

In all these cases as well as in M. violagabriellae iron may act as a catalytic agent in the oxidative processes leading to the formation of the polymerized phenolic compound.

Elemental analysis of the pigment revealed the presence of C, H, N and S. C, H and N have been found in melanins by Kertesz (1957) and Raper (1927). Rothman and Flesch (1943) found human red hair melanin to contain 45.16 per cent C, 4.96 per cent H, and 7.21 per cent N. These figures, although similar, differ from those found for M. violagabriellae pigment. This may be due to the presence of the peptide and sugar component attached to the pigment, and/or the result of alkaline hydrolysis of the pigment during its preparation for analysis.

The UV and visible spectrum of the pigment is dissimilar to that reported for most melanins, since the latter are not characterized by resolved electron band spectra but by general absorption and possible scattering effects (Mason, 1948). It is for this reason that color cannot in itself be a distinguishing criterion for melanin.

Bowness and Morton (1953) investigated the spectrum of dopa-melanin in water and found it to be characterized by general absorption in the region 250-700 mu, with a small peak at 280 mu. Even when Co, Cu and Fe were added to thse solutions, the spectrum still showed inflexions at 280 mu.

Mason (1949) followed the spectrum of oxidation of catechol and hydroquinone by tyrosinase and reported that the final stages of oxidation were characterized by inflections or maxima in the region of 255 to 270 mu.

The enzymic oxidation of dopa was followed by Mason (1948). The reaction proceeded in three chromophoric phases characterized by absorption spectra with maxima at a) 305 and 475 mu, b) 300 and 540 mu and c) general absorption.

Stein (1954) compared the spectra of natural and synthetic melanins. Melanin from the choroid of the ox-eye and auto-oxidized dopa showed maxima at 270 mu, whereas enzymic oxidation of tyrosine exhibited a peak at 285 mu.

Stein (1955) showed choroid-melanin, black hair

melanin and red hair melanin spectra all had inflections in the region of 270 mu.

The pigment of M. violagabriellae is similar to these in the presence of a peak at 284 mu, but is not characterized by general absorption. However, the absorption spectra of pigments of T. rubrum (Baichwal and Walker, 1960) are similar to those of M. violagabriellae in the presence of maxima at approximately 280 and 400 mu. But, there is no corresponding peak in the region 227 to 246 mu.

The IR spectra of different melanins (Figure XIX) (page 92) prepared by Bonner and Duncan (1962) all show characteristic absorptions which can be attributed to N H (or OH) bonds at 3 u and carbonyl groups at 6 u. Differences do exist between these melanins.

The pigment of M. violagabriellae exhibits areas of absorbance in both these areas. The peak at 2950 cm^{-1} may be due to CH bonds; 1650 cm^{-1}, an amide linkage; 1515 cm^{-1}, a C=C of an aromatic ring; 1465 cm^{-1}, and 1393 cm^{-1}, CH_2 groups; and maxima between 1217 cm^{-1} and 700 cm^{-1} could be C-O bonds; 845 cm^{-1} may indicate a tetrasubstituted compound.

This data would support the idea of an aromatic system with a peptide component, and free NH_2 or OH groups. Because of the strength of the signal between 13 and 15 u, the molecule may be a large one.

Rangier (1959) studied three melanins using infrared spectroscopy, the results of which are listed below (frequencies expressed as cm^{-1}).

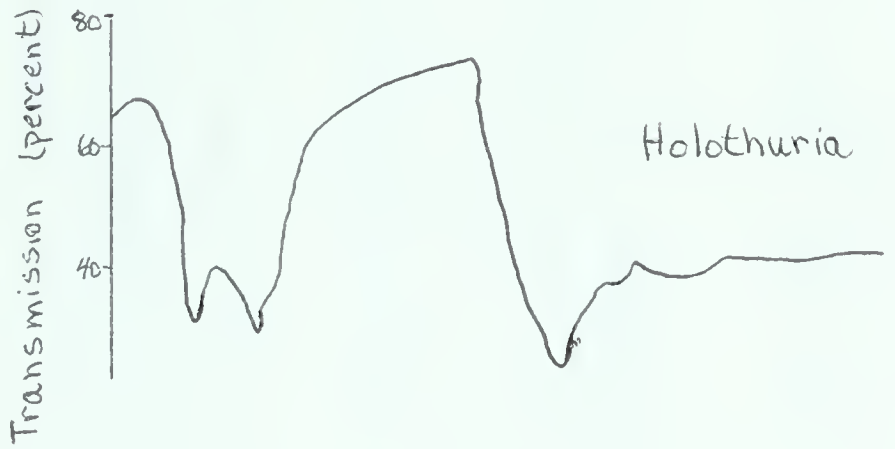
FIGURE XIX

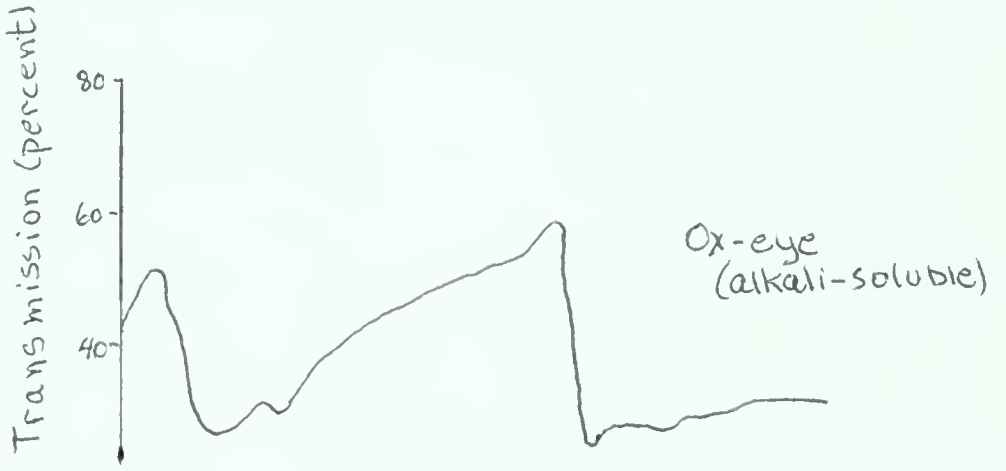
IR Spectra of Some Melanins

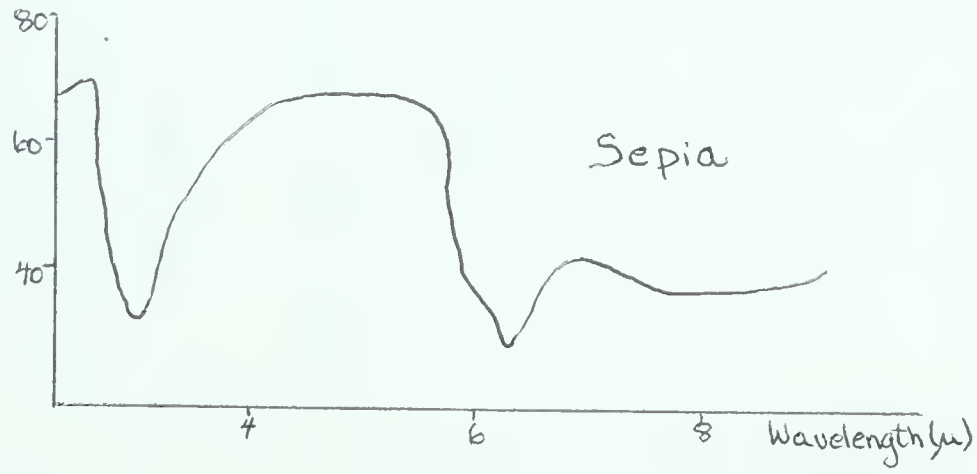
(Corresponding to Fig. I of Bonner and Duncan, 1962)

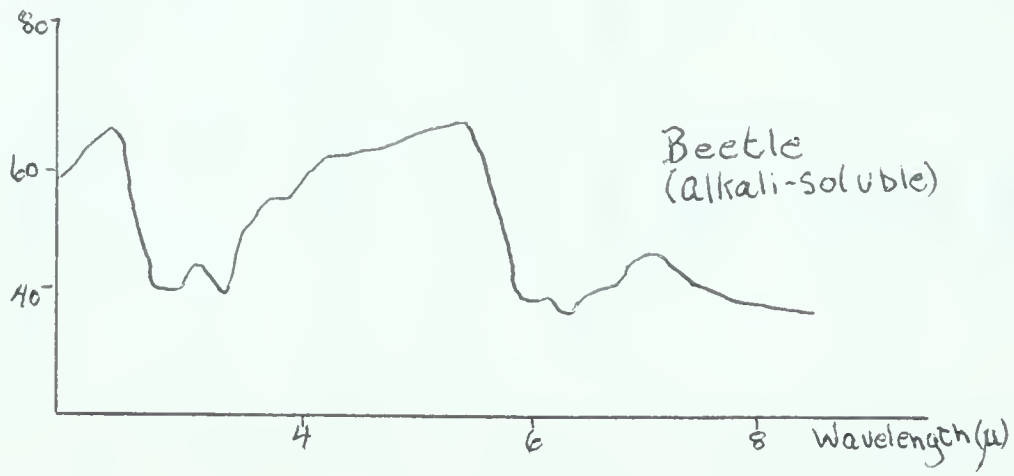

80
60
40
Arenicola


80
60
40
Ox-eye
(alkali-insoluble)


Transmission (percent)
80
60
40
Holothuria


Transmission (percent)
80
60
40
Ox-eye
(alkali-soluble)


80
60
40
Sepia
4
6
8
Wavelength (μ)


80
60
40
Beetle
(alkali-soluble)
4
6
8
Wavelength (μ)

	Adrenochrome	Hair	Tumor
CO	-	-	1206
COC	-	1615	1625
COO	1457	-	1459
COOH	1250	-	-
CN	-	1897	1920

This data also illustrates the differences existing between IR spectra obtained from different melanins. Rangier makes no mention of maxima around 3 u (3350 cm^{-1}), characteristic of the spectra of M. violagabriellae pigment and those published by Bonner and Duncan (1962).

The presence of maxima at 1897 and 1920 cm^{-1} in two of the melanins attributable to CN linkages are decidedly different from the isolated pigment of M. violagabriellae and those of Bonner and Duncan (1962).

The IR spectra of pulcherrimin (MacDonald, 1963) has some maxima in common with the isolated pigment. Pulcherrimin has maxima at approximately 1500, 1425, 1400, 1375, 1340, 1280, 1225, 1140, 1075, 1015, 975, 925, and 840 cm^{-1}.

Absorption spectra of M. violagabriellae pigment in ethylene chlorohydrin (after alkali-acid treatment) is different from that of the pigment in N KOH. This fact would indicate that a hydrolysis of the pigment had taken place in the alkali.

Further evidence of alkaline hydrolysis was substantiated by the spectral changes of the pigment, the loss of iron from the molecule to form an insoluble iron

oxide, the change in color and quantitative reduction of the pigment which could be precipitated by acid from alkaline solution, and changes in the solubility of the pigment following alkali-acid treatment.

The pigment of O. nigra (Fontaine, 1962) was more soluble after extraction by alkali, which would suggest that a considerable amount of alkaline hydrolysis had taken place in this melanin.

Gortner (1910) studied the effect of alkali on melanin from black wool. He found that, except in small concentrations, alkali readily destroyed the greater portion of the melanin molecule. The nitrogen and hydrogen content dropped rapidly, the sulfur content remained constant and the carbon and oxygen contents increased as the molecule was broken down under the influence of increasing strengths of sodium hydrate.

The 'white' pigment obtained by acid precipitation from alkaline solution still contained a peptide and pentose component and these components must be relatively insusceptible to alkaline hydrolysis. The UV spectra illustrates the decomposition of this pigment which had taken place prior to acid precipitation. The IR spectra of the 'red' and 'white' pigments were similar. In the latter case, there was no peaking from 1100 to 700 cm^{-1}.

The iron compound which was precipitated from the pigment in alkaline solution appeared to be an iron oxide. It was free from amino acid and sugar components and exhibited no IR maxima.

Pigment isolated from cells grown in the absence of iron was similar to that of the red pigment, but some reduction in the intensity of the peaks in the IR region did occur. Reduction of ammoniacal silver nitrate was not possible with this pigment.

Two respiratory poisons, NaN_3 and KCN, inhibited pigmentation but not growth in the concentrations used, whereas NaF had no deliterious effect on pigmentation. These studies would indicate the involvement of the cytochrome system, since both azide and cyanide are known inhibitors of cytochrome oxidase.

Kopac (1948) reports that dopa can be oxidized by cytochrome oxidase and cytochrome c.

Hermann and Boss (1945) reported an increase in the oxidation of dopa, catechol, and hydroquinone on addition of cytochrome c, which indicated the presence of cytochrome oxidase.

Burk et al. (1948) reported that cytochrome oxidase catalyzed the oxidation, by molecular oxygen, of p-phenylenediamine.

Two enzymic pathways were shown for the in vitro production of melanin from dopa by Hesselbach (1951). These were the phenolase and cytochrome oxidase systems.

Although the cytochrome system may play a role in pigment synthesis by M. violagabriellae, the existence of a phenolase is suggested by inhibition of pigmentation by p-cresol, o-cresol, m-cresol, and p-benzyloxyphenol.

The latter compound is a known inhibitor of melanogenesis (Lerner and Fitzpatrick, 1950) in mammalian systems.

Mencher and Heim (1962) reported that p-benzyloxyphenol inhibited melanin biosynthesis in Streptomyces lavendulae with concentrations that did not inhibit cell synthesis.

The action of tyrosinases from different sources on various phenolic compounds such as p-cresol to form polymerized products is well known (Fox, 1953). It is surprising therefore to note the p, m, o-cresols all act as pigment inhibitors in M. violagabriellae.

Inhibition by glutathione (reduced) is also characteristic of other systems. Figge and Allen (1941) found that glutathione-inhibited melanin formation could be released by estrone.

Kohn (1955) found that glutathione inhibited melanin synthesis from tyrosine by the action of tyrosinase in vitro. When dopa was used as the substrate, glutathione inhibited by combination directly with the enzyme rather than by reducing a substrate intermediate.

Cysteine has been reported to act in an analogous manner (Figge and Allen, 1941) but inhibition could not be achieved using cysteine in M. violagabriellae. This may be due to the fact that cysteine is quite readily metabolized (Chapter I).

Ascorbic acid in melanin producing reactions inhibits by acting as a reducing agent. The addition of ascorbic

acid to a tyrosinase-tyrosine system increased the accumulation of dopa by reduction of dopa-quinone (Evans and Raper, 1937).

Zussman et al. (1959) reported that in experiments with T. rubrum, ascorbic acid was able to decrease or prevent pigment formation when added to the medium in concentrations that did not seriously inhibit growth.

Besides ascorbic acid, pigmentation in M. viola-gabriellae could be inhibited by methylene blue. Methylene blue as well as other dyes have been reported to produce pallor in experimental animals (Figge, 1948).

The brown compound which accumulates when pigmentation in cultures was inhibited by p-cresol was very similar in UV and IR spectra and solubilities to the melanins produced by the 2 strains of Pseudomonas studied. These compounds appear to be very closely related.

The IR spectra of the melanins from Pseudomonas aeruginosa M 117 and MS strains were almost identical, as was their solubility characteristics. These pigments are probably identical.

The pigment isolated has definite melanin-like characteristics but is different from classical melanin-synthesis systems in that a dependence on tyrosine could not be demonstrated, inhibition reversal could not be accomplished using tyrosine or shikimic acid, and the presence of dopa could not be demonstrated.

The isolated pigment is also similar to pulcherrimin in the dependence of iron for red pigmentation, solubility characteristics, removal of iron in alkaline solution, and precipitation from alkaline solution by the addition of acid.

The pigment is unlike the melanin pigments isolated from Pseudomonas, which are in turn dissimilar to classical melanins in view of their solubility in water.

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